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(54) **Cloning system for Kluyveromyces species.**

(57) A new cloning system is described capable of expressing genetic material derived from recombinant DNA material, which comprises a yeast of the genus *Kluyveromyces* as a host. Suitable vectors are e.g. vectors containing autonomously replicating sequences (ARS) and vectors containing homologous *Kluyveromyces* DNA acting as a site for recombination with the host chromosome. New and preferred vectors are those containing ARS sequences originating from *Kluyveromyces* (KARS vectors). The genetically engineered new strains of *Kluyveromyces* produce, inter alia, lactase and chymosin.

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CLONING SYSTEM FOR KLUYVEROMYCES SPECIES

Field of the invention

This invention relates to the field of recombinant DNA biotechnology. It particularly relates to the use of recombinant DNA biotechnology in the production of polypeptides. More particularly, the present invention relates to new recombinant DNA cloning vehicles and suitable host organisms therefor, which can be used for the high yield production of polypeptides, e.g. enzymes such as beta-galactosidase (lactase) and chymosin and its precursors.

Background of the invention

In the past few years, microorganisms have proved to be capable of producing foreign peptides and proteins, encoded by foreign genes artificially introduced by means of a transformation system and expressed under the control of regulatory sequences.

Some of the basic techniques for this procedure have been disclosed in, for example, US patent 4,237,224.

The basic constituents of recombinant DNA technology are formed by:

- the gene encoding the desired property and provided with adequate control sequences required for expression in the host organism,
- a vector, usually a plasmid, into which the gene can be inserted to guarantee stable replication and a high level of expression of the said gene,
- a suitable host microorganism in which the vector carrying the said gene can be transformed and having the cellular systems to express the information of the said gene.

Amongst the products thus formed are enzymes, hormones, antigens and other useful peptides and proteins.

Some of these products are used as pharmaceutical agents, e.g. growth hormone and interferon, others as auxiliaries in the food industry e.g. beta-galactosidase (lactase), chymosin and amyloglucosidase, and still others may act as biological catalysts for the conversion of certain compounds.

Every contamination of pharmaceuticals or food with harmful organisms or substances should be excluded. The host organisms should also be harmless to persons handling the microbes during experimentation or large scale production processes. Therefore, a prerequisite for the host is that it is not pathogenic.

The first years of recombinant-DNA work were characterized by stringent rules and restrictions to prevent or limit undesired side effects, especially the uncontrolled dissemination of pathogenic microorganisms in the environment.

Although the concern about the supposed risks seems to have been exaggerated, there still remains a steady need for hosts which are not associated with any noxious effect.

Up to now, commercial efforts involving recombinant genetic manipulation of plasmids for producing various substances have centered on Escherichia coli as a host organism. The main reason is that E. coli is historically the best studied microorganism. The first discoveries and inventions made in recombinant DNA technology have been made with E. coli as the host.

However, E. coli is not the most desirable organism to use for commercial production of substances applied in pharmaceutical and food industry. It may even prove to be unsuitable as a host/vector system in some situations, because of the presence of a number of toxic pyrogenic factors. The elimination of these may often cause problems. Therefore, E. coli has only a very limited use as production organism in fermentation industry. Also the proteolytic activities in

E. coli may seriously limit yields of some useful products.

These and other considerations have led to an increased interest in alternative host/vector systems. The interest is concentrating in particular on the use of eukaryotic systems for the use of eukaryotic products. A continuing demand exists for new hosts which are above any suspicion as production organisms for chemical substances, in particular food-grade and pharmaceutical grade products, and which moreover are suited to large scale fermentations in industry.

The names of many harmless microorganisms are found on the so called GRAS (Generally Recognized as Safe) list. However, only few genetic procedures are known sofar for the cloning and expression of genes in GRAS-organisms.

Amongst the eukaryotic organisms suitable for commercial exploitation yeasts are perhaps the easiest ones to manage. Yeast, especially bakers' yeast, is relatively cheap, easy to grow in large quantities and has a highly developed genetic system.
The term yeast is frequently used to indicate only Saccharomyces cerevisiae or bakers' yeast, which is one of the most common and well-known species. It will be understood that the term yeast as used in this specification is meant to indicate all genera and is not restricted to the species Saccharomyces cerevisiae.

Recently, it has been disclosed that cells of Saccharomyces cerevisiae are susceptible to transformation by plasmids (A. Hinnen et al., Proc. Natl. Acad. Sci. USA 75 (1978), 1929. Success has been had with cloning and expressing in this yeast the bacterial resistance genes for ampicillin, chloramphenicol and kanamycin, but also eukaryotic genes like the lactase gene and the heterologous genes for ovalbumin, leukocyte interferon D and also a Drosophila gene (see review C.P. Hollenberg, Current Topics in Microbiology and Immunology, 96 (1982) 119-144).

Up to now, only one other yeast species has been investigated as a host for yeast expression vectors. The Saccharomyces cerevisiae leucine gene has been successfully cloned and expressed in Schizosaccharomyces pombe (D. Beach, and P. Nurse, Nature 290 (1981) 140-142).

Yeast vectors which have been described to give successful transformation are based on the natural 2 μm (2 micron) plasmid occurring in many strains of S. cerevisiae (see e.g. J.D. Beggs, Nature 275 (1978), 104-109), and on the autonomously replicating sequences (ARS) derived from yeast chromosomal DNA (see e.g. K. Struhl et al., Proc. Natl. Acad. Sci. USA 76 (1979), 1035-1039), respectively. Vectors for Saccharomyces cerevisiae which can be used for transformation purposes have also been described by C.P. Hollenberg, Current Topics in Microbiology and Immunology, 96 (1982) 119-144.

The transformation of not well characterized or industrial yeast species is severely hampered by the lack of knowledge about transformation conditions and suitable vectors. In addition, auxotrophic markers are often not available or are undesired, precluding a direct selection by auxotrophic complementation.

Summary of the invention

It is an object of this invention to provide a yeast vector system capable of expressing an inserted polypeptide coding sequence.

It is a further object of this invention to provide new genetically engineered yeast strains of the genus Kluyveromyces which are capable of producing polypeptides in culture for mass production.

Is is another object of this invention to provide new genetically engineered yeast strains of the genus Kluyveromyces

which are capable of producing chymosin and its precursor forms in culture for mass production.

It is still a further object of this invention to provide new genetically engineered yeast strains of the genus Kluyveromyces which are capable of producing lactase in culture for mass production.

It is still another object of this invention to provide processes for the manufacturing of polypeptides and in particular enzymes with Kluyveromyces as producing organism obtained by recombinant DNA techniques.

It is still another object of this invention to provide particular modified Kluyveromyces cells for use in production of polypeptides displaying certain enzymatic activities.

These and other objects will be described in more detail in the further specification.

Description of the invention

Yeasts of the genus Kluyveromyces and in particular the species K. lactis and K. fragilis are biotechnologically important and are of commercial interest. Kluyveromyces lactis and Kluyveromyces fragilis, for example, are used for the commercial production of the enzyme lactase (beta-galactosidase). Kluyveromyces organisms are mentioned on the GRAS-list.

In contrast with most of the bacterial species investigated in transformation experiments, yeast cells possess a cell wall impermeable for plasmids. Therefore, a usual preparatory step of yeast transformation is the removal of the cell wall, yielding protoplasts which are able to take up plasmids. Cell wall lytic enzymes which advantageously may be used are selected from the group of beta-1,3-glucanases. A suitable example is helicase, a crude enzyme preparation

originating from gut of the snail Helix pomatia. Another suitable representative is zymolyase.

It is well known that the cell wall can be regenerated during subsequent incubation under suitable conditions. However only a fraction of the protoplasts regenerates and for Kluyveromyces lactis this process has appeared to be even twenty times less efficient than for Saccharomyces cerevisiae under similar conditions.
Although transformation of yeasts using protoplasts has been decribed by several authors, it appears that some yeast strains and particularly wild type yeast strains and Kluyveromyces species are very difficult to regenerate.
Hollenberg described (Current Topics in Microbiology and Immunology, 96 (1982) 119-144), how the regeneration of protoplasts of Saccharomyces cerevisiae can also take place if the usual osmotic stabilizer sorbitol is substituted by 0.6 M potassium chloride. It has now been surprisingly found that by applying this method to Kluyveromyces protoplasts the fraction of regenerated yeast cells even increases three to fivefold.

Recently, a method has been disclosed by Ito et al (J. Bacteriol. 153 (1983) 163-168), in which whole cells are used instead of protoplasts, thereby circumventing the regeneration step. This method has been shown to be effective in S. cerevisiae with certain types of plasmids.
It has now been found that this method is surprisingly effective in Kluyveromyces, particularly when plasmids containing KARS-sequences (as will be described hereinafter) are used.

It will be appreciated by those skilled in the art, that the availability of a suitable vector is of decisive importance. Beforehand it is uncertain if a specific host/vector combination will act successfully as a transformation system. For example, it is known from E. Erhart and C.P. Hollenberg, Current Genetics 3 (1981) 83-89, that plasmid pMP81 can be

transformed into Saccharomyces cerevisiae YT6-2-IL (cir°) but not into Kluyveromyces lactis. D. Beach and P. Nurse disclosed in Nature 290 (1981) 140-142, that plasmid pJDB219 has a high copy number in Saccharomyces cerevisiae, but transforms Schizosaccharomyces pombe at the very low frequency of only 2 transformants per microgram DNA.

Up to now vectors for Kluyveromyces were not known at all.

As a result of extensive research and experimentation new vectors were found which are capable of transforming the host organism Kluyveromyces and which, moreover, are able to replicate autonomously in the transformed cell.

The new vectors, which are particularly suitable for Kluyveromyces and preferably for K. lactis and K. fragilis, can be distinguished in two categories according to the constituting DNA sequences which control the function of replication and maintenance in, for example, Kluyveromyces species viz.:

1. vectors containing autonomously replicating sequences (ARS), and
2. vectors devoid of autonomously replicating sequences but containing homologous Kluyveromyces DNA acting as a site for recombination with the host chromosome.

Suitable and preferred ARS vectors are those originating from Kluyveromyces, also referred to as KARS vectors. Said vectors of the KARS type are preferably used because of their high transformation frequency. Vectors of the second category usually transform with low frequency but they are very stably retained in the host cells.

Preferred vectors of the first category are, for example, KARS vectors originating from K. lactis, of which pKARS12 and pKARS2 are the most preferred. pKARS12 and pKARS2 are hybrid plasmids composed of a K. lactis DNA fragment containing the KARS12 and KARS2 sequence, respectively which

are inserted into the known S. cerevisiae plasmid YRp7.

A preferred vector of the second category is, for example, pL4, a hybrid plasmid composed of the ARS1 carrying plasmid YRp7 and a K. lactis XhoI DNA fragment carrying the LAC4 gene.

For transformation purposes in Kluyveromyces the following genes can, for example, be advantageously used as selectable markers on the vectors:

1. the tryptophan gene (TRP1) derived from S. cerevisiae;
2. the lactase gene (LAC4) derived from K. lactis;
3. the Kan^R gene coding for resistance against the antibiotic G418 which is related to gentamycin, derived from E. coli.

On the vectors there are suitable restriction sites which allow further gene cloning.

The stability of the transformed plasmids may considerably be enhanced if a centromer region (CEN) from the K. lactis or S. cerevisiae chromosome is inserted in the vector.

Also Escherichia coli is a suitable host, especially for cloning and storage. In that case the ampicillin resistance gene (Amp^R) is also a suitable selectable marker on the vector. The plasmids are preferably multiplied and stored within E. coli cells, particularly those of the strains DG75 and JA221. The transformed strains are selectively grown on L-broth containing:

kanamycin (20 µg/ml) for E. coli DG75 (PTY75-LAC4, and ampicillin (100 µg/ml) for E. coli DG75 (pL4) and E. coli JA221 (pKARS12).

Said transformed strains were deposited under Rule 28, resp. 28a of the European Patent Convention with the Centraal Bureau of Schimmelcultures, Oosterstraat 1, 3742 SK Baarn, the Netherlands under numbers CBS 351.82 (=LMD 82.18), CBS

352.82 (=LMD 82.19) and CBS 353.82 (=LMD 82.20), respectively, on 19th May 1982. The plasmids can be isolated from the cells, e.g. by the method of L. Katz et al., J. Bacteriol. 114 (1973) 577.

The protoplasts of the yeast host are transformed by the aforementioned vectors in a usual incubation medium containing Tris, calcium chloride and polyethylene glycol having a molecular weight ranging from 2000 to 6000, but preferably of 4000.

Prokaryotic transformants can easily be detected by well-known means of primary selection. Even if the desired property is not recognizable in the phenotype of the transformant, the vector usually contains one or more genes coding for primary selectable properties like antibiotic resistance or genes coding for essential growth factors. In the latter case the corresponding auxotrophic mutant of the host should be available. While there are many auxotrophic prokaryotes available, the number of auxotrophic industrial yeasts is limited. Mutation of a gene from a production strain often adversely affects important growth and production characteristics of that strain.

The transformation method according to this invention, using whole cells instead of protoplasts for the transformation of Kluyveromyces species, can be suitably performed as follows.

The method according to the invention comprises growing Kluyveromyces cells in standard yeast medium and harvesting the cells at OP_{610nm} between 1 and 25. Optimal results are obtained at OD_{610nm} between 4 and 10.
The Kluyveromyces cells are washed and preheated with certain types of chaotropic ions, e.g. Li^+, Cs^+, Rb^+. LiCl and Li_2SO_4 are conveniently used, at final concentrations of about 20mM-0.2M, preferably about 0.1M.
The Kluyveromyces cells are incubated with said monovalent ions at 30°C for about 5-120 minutes, usually about 60 min.

followed by an incubation with DNA. The transformation can be enhanced if subsequently polyethylene glycol is added. Generally, an equal volume of 70% polyethylene glycol 7000 is used. The Kluyveromyces transformation can be further enhanced by exposing the cells to a heat treatment. For example, by a treatment for about 5 minutes at about 42°C, the enhancement is about 20-fold.
The use of this procedure according to the invention will be shown in detail in the Examples with Kluyveromyces lactis SD11, Kluyveromyces fragilis leu 24 and Kluyveromyces fragilis C12 as respective host organisms.

In contrast to prokaryotes, the use of antibiotic resistance markers in yeast is far from easy. Only a small number of antibiotics is active against yeast. Moreover, the resistance factors predominantly originate from bacteria and it is not at all obvious if they can be expressed in yeast cells and used as a selective marker.
Kanamycin and the aminoglycoside G418 which is related to gentamycin have been shown to be poisonous for cells of wild type yeast strains.

It is further known from Hollenberg, Extrachromosomal DNA, ICN-UCLA Symp. (1979) 15:325-338, Acad. Press, New York, that the transposable resistance element Tn601 (present on bacterial plasmid pCR1) contains a gene that confers resistance to kanamycin to transformants of Saccharomyces cerevisiae. A. Jimenez and J. Davis, Nature 287 (1980) 869-871, showed later that the kanamycin resistance gene can also confer resistance to S. cerevisiae transformants against antibiotic G418, a potent inhibitor of yeast growth.

The plasmid PTY75-LAC4, a hybrid plasmid composed of the plasmid pCR1, the 2 μm plasmid from S. cerevisiae and the Sal I fragment from plasmid pK16, carrying the K. lactis LAC4 gene and forming also a feature of the present invention, contains the same resistance gene. It has now been found that this gene is expressed also in Kluyveromyces

lactis, enabling the strain to inactivate G418 taken up from the growth medium and providing thus a tool for primary selection of Kluyveromyces lactis transformants.

Although plasmid PTY75-LAC4 does not contain any autonomously replicating sequence from Kluyveromyces, it was surprisingly found that plasmids containing the 2 μm plasmid from S. cerevisiae, such as PTY75-LAC4, do replicate autonomously in Kluyveromyces species.

Selection of G418 resistant yeast cells transformed by PTY75-LAC4 was performed on regeneration plates containing glucose, sorbitol and 0.2 mg/ml G418. KCl is not suited here because, dueg to high salt concentration, Kluyveromyces lactis cells are insensitive to G418, even in concentrations up to 0.8 mg per ml.

Resistant colonies appear within 5-6 days after transformation with PTY75-LAC4. Real transformants can be distinguished from colonies which have become resistant by spontaneous mutation by checking the presence of PTY75-LAC4 DNA by colony hybridisation with labelled pCR1 DNA, or, in case a K. lactis lac4 mutant is used as host strain, by checking their ability to grow on minimal medium (yeast nitrogen base, Difco) with lactose as the sole carbon source. On the average, 5% of the resistant colonies were found to contain PTY75-LAC4 DNA or to be Lac^+. By this method of selection about 4 transformants per microgram of plasmid DNA were obtained.

Direct selection in K. lactis SD69 lac4 for the presence of the LAC4 gene, using plates containing lactose as sole carbon source and 0.6M KCl as osmotic stabilizer, yielded 20 Lac^+ transformants after 4 to 5 days of incubation at 30°C. On control plates without DNA no Lac^+ colonies were found to appear within said period. The Lac^+ colonies of the direct selection were shown to be transformants and not spontaneous revertants, because the presence of the KanR marker on

G418 plates could be demonstrated as described above.

When plasmid pL4 or KARS-type plasmids are used, one also has the possibility of selecting for the presence of tryptophan prototrophy in the transformants. In comparison with plasmid PTY75-LAC4, the use of plasmid pL4 caused a substantial increase in the efficiency of transformation: 30 transformants per microgram DNA were found. The KARS-type plasmids, however, having 10^3-10^4 transformants per microgram DNA appear to be far superior.

The plasmid PTY75-LAC4 and KARS-containing plamids were found to exist in transformed cells autonomously replicating. This was demonstrated with the aid of DNA analysis. Undigested minilysates of transformants were analyzed according to the Southern blot procedure, by hybridization with ^{32}P-labelled pCR1, the bacterial component of plasmid PTY75-LAC4 or with labelled pBR322, the bacterial part of the pKARS plasmids.

Comparative electrophoresis of a minilysate of an untransformed Kluyveromyces lactis lac4 mutant and of purified plasmid preparations shows that only in the transformants hybridizing bands are present with electrophoretic mobilities corresponding to supercoiled and open circular forms of the plasmid used for transformation.

Presence of the plasmid in transformed cells was further confirmed by transforming E. coli with the DNA preparation from the yeast transformants and isolating the same plasmids from the E. coli transformants formed.

The process of the present invention can be applied to host strains of the species Kluyveromyces lactis as well as to strains of the species Kluyveromyces fragilis. Both species are safe organisms and appear on the GRAS-list.

Particularly useful hosts are the mutants Kluyveromyces

lactis SD11 lac4 trp1 and SD69 lac4 which are derived from the wild type CBS 2360 and deposited under Rule 28, resp. 28a of the European Patent Convention with Centraal Bureau voor Schimmelcultures, Oosterstraat 1, 3742 SK Baarn, Netherlands, under numbers CBS 8092 and CBS 8093, respectively, on 19th May 1982.
Usually, transforming plasmids remain within the host cell as separate entities capable of autonomous replication and expression. It is pointed out here, however, that genes, after having been introduced on plasmids (with or without replication sequences) can subsequently also be integrated in the chromosomal DNA of the cell.

This so-called integrative transformation appeared to have occured in stable K. lactis SD11 trp1 Lac^+ transformants after transformation with plasmid pL4. In this case no free plasmid DNA is present in the transformants.
Integration of the LAC4 gene can be demonstrated by Southern blot DNA analysis of the total cell DNA that is digested by restriction enzymes, the pL4 plasmid functioning as a labelled hybridization probe.

To maintain the plasmids in the yeast transformants the following selective media can be used, for example: yeast nitrogen base medium (DIFCO) plus 2% lactose instead of glucose for K. lactis SD69 lac4 (PTY75-LAC4) and for K. lactis SD69 lac4 (pL4) and yeast nitrogen base medium (DIFCO) plus 2% glucose for K. lactis SD11 trp1 lac4 (pKARS12).

Hybrid plasmids have been constructed consisting of KARS12-LAC4 and KARS12-2 μm DNA-LAC4 sequences.
When the new microorganisms according to the invention are used for large scale production, it is desirable to remove all bacterial DNA sequences from the vector plasmids.

Genes can remain on autonomously replicating plasmids after having been introduced into the cell or may be integrated in

the chromosomal DNA of the host cell.

The invention can be used for the cloning and expression of both prokaryotic and eukaryotic genes in Kluyveromyces as a host, preferably using a plasmid vector of one of the types as described hereinbefore. Suitable prokaryotic genes for use according to the invention are, for example, lactase, alpha-amylase, amyloglucosidase and beta-lactamase. Suitable eukaryotic genes for use according to the invention are, for example, lactase, chymosin, invertase and interferon. For the insertion of the genes coding for these products suitable restriction sites are available on the vectors as described hereinbefore.

According to this invention, prokaryotic and eukaryotic genes, both homologous and heterologous, can be used. The invention can advantageously be used for the high production of chemical substances, in particular polypeptides. A preferred embodiment of the invention is the production of chymosin, a milk clotting enzyme.

The choice of the vector and regulons for the cloning and expression of genes in Kluyveromyces may, of course, vary with the gene used in a particular case. Also, the choice of a particular Kluyveromyces strain as a host and the optimal process conditions may vary with, inter alia, the gene and vector to be selected. The optimal selection and process conditions can be established by routine experimentation. These variations are all included within this invention.

The invention is further exemplified by a detailed description of the cloning and expression of:

a. a homologous gene, beta-galactosidase (lactase) in K. lactis;
b. a prokaryotic heterologous gene, Kan^R, in K. lactis and K. fragilis;
c. a eukaryotic heterologous gene, TRP1, in K. lactis;

d. a eukaryotic heterologous gene, LEU2, in K. fragilis;
e. a eukaryotic heterologous gene, encoding preprochymosin and its maturation forms, in K. lactis; and
f. a eukaryotic heterologous gene, encoding preprothaumatin and its maturation and modified forms, in K. lactis.

The following Examples illustrate certain embodiments of the present invention.

Example 1

Recombinant plasmid PTY75-LAC4

0.5 µg of the plasmid pK16 described by R. Dickson, (Gene 10 (1980) 347-356) and 0.5 µg of the plasmid PTY75 described by C.P. Hollenberg et al. (Gene 1 (1976) 33-47) were digested with the restriction enzyme Sal I. The two digests were mixed and after inactivation of the restriction enzyme the solution was incubated with T4-ligase, yielding a solution with recombinant DNA.

This ligated mixture was used to transform to the E. coli strain DG75 (hsdS1 leu-6 ara-14 galK2 xyl-5 mt-1 rpsL20 thi-1 supE44- λ^--lac Δz 39) according to R.C. Dickson et al., Cell 15 (1978) 123-130, resulting in kanamycin resistance (Kan^R). Kan^R colonies were further selected on supplemented minimal plates, containing lactose as the sole carbon source, for the formation of lac^+ colonies. The plasmid PTY75-LAC4 was isolated from one of the selected Kan^R lac^+ transformants, using the method according to L. Katz et al., J. Bacteriol. 114 (1973) 577-591.

Example 2

Recombinant pKARS plasmids

5 g of plasmid YRp7 (Struhl et al., Proc. Natl. Acad. Sci., 76 (1979) 1035-39) were digested with restriction enzyme Sal I. 14 µg of DNA from the wild strain K. lactis CBS 2360 were digested with enzyme Xho I. The fragments of the plasmid and the K. lactis DNA were mixed in a molar ratio of 1:3. After inactivation of the restriction enzymes the solution was brought to a DNA concentration of 25 µg/ml and incubated with T4-ligase under standard conditions (Boehringer).

Transformation of E. coli DG75 with the ligated mixture under usual conditions yielded a mixture of 4.5×10^5 Amp^R transformants, 9×10^3 of which contained K. lactis inserts, as can be deduced from their sensitivity to tetracyclin. The proportion of tetracyclin-sensitive cells can be increased to 85% by cycloserine treatment, see F. Bolivar and

K. Backman, Methods in Enzymology 68 (1979) 245-267. According to the method of Katz et al. (see Example 1) 14 different plasmids were isolated, which are referred to as pKARS 1-14. All were capable of transforming K. lactis SD11 lac4 trp1 strain to Trp^+ phenotype with a frequency of 10^3-10^4 per microgram of DNA. Plasmid pKARS12 showed the highest transformation frequency of $3x10^4$ per microgram of DNA, but plasmid pKARS2 appeared to be more convenient in further processing.

The recombinant plasmids obtained could also be transferred to E. coli JA221 (Δtrp E5, leu B6, lac y, rec A, $hsdM^+$, $hsdR^-$).

Example 3

Recombinant plasmid pL4

A mixture of YRp7 and K. lactis DNA fragments was prepared as described in Example 2. E. coli DG75 strain was transformed with the ligated mixture and subsequently plated on M9 minimal agar, the medium of which contained lactose as the sole carbon source, to which leucine had been added. Lac^+ colonies appeared after 8 days at 30°C. Plasmid pL4 was isolated from one of these lac^+ colonies using the method of Katz et al. (see Example 1).

Example 4

Kluyveromyces lactis SD69 lac4 transformed to $G418^R$ $lac4^+$ with plasmid PTY75-LAC4

Cells of the Kluyveromyces lactis mutant SD69 lac4 were suspended in a growth medium (pH 6.8) containing 1% of yeast extract, 2% of peptone and 2% of glucose. The growth was continued until the exponential phase ($3-5.10^7$ cells per ml) had been reached.

The yeast cells were collected by centrifugation, washed with water and resuspended in a solution (pH 8.0) containing 1.2M sorbitol, 25mM EDTA and 0.2M fresh mercaptoethanol.

After incubation for 10 min. at 30°C the cells were centrifuged, washed two times with a 1.2M sorbitol solution and resuspended in 20 ml of a solution (pH 5.8) containing 1.2M sorbitol, 10mM EDTA, 0.1M sodium citrate and 10mg helicase.

Protoplasts were formed and after 15-20 min. these were centrifuged, washed three times with 1.2M sorbitol and resuspended to a concentration of about 5.10^{10} cells per ml in 0.1 ml of a solution containing 10mM $CaCl_2$ and 1.2M sorbitol.

10 µg of pTY75-LAC4 were added and the mixture was incubated for 15 min at 25°C. Thereafter 0.5 ml of a solution (pH 7.5) containing 10mM Tris, 10mM $CaCl_2$ and 20% (w/v) polyethylene glycol 4,000 was added, followed by 20 minutes incubation.

Protoplasts were precipitated by centrifugation and then resuspended to a concentration of about 5.10^{10} protoplasts per ml in a solution (pH 6.8) containing 7 mM $CaCl_2$, 1.2M sorbitol, 0.5 mg/ml yeast extract, 1 mg/ml peptone and 2 mg/ml glucose.

After incubation for 60 min. at 30°C the protoplasts were centrifuged, washed with 0.6 M KCl solution and resuspended in 0.6 M KCl solution.

In order to be able to select the G418 resistant transformants, 1.10^9 protoplasts were plated in a 3% agar overlay on 2% minimal agar plates containing 2% of glucose, 1.2M sorbitol and 0.2 mg/ml of the antibiotic G418. For the purpose of simultaneously selecting Lac^+ transformants, 5.10^8 protoplasts were plated in 3% agar overlay on 2% minimal agar plates, DIFCO yeast nitrogen base medium, containing 2% lactose as the sole carbon source and 0.6M KCl instead of 1.2M sorbitol.

Colonies appeared within 4-5 days. On sorbitol plates without G418 protoplast regeneration was usually 0.2-0.5%,

whereas on the 0.6M KCl plates with glucose as carbon source this percentage increased to 0.5-1.5%.

When G418 was used for the selection, one transformant was obtained per 10^7 regenerated protoplasts. Simultaneous selection on lactose plates yielded 10 transformants per 10^7 regenerated protoplasts or 20 transformants per microgram of plasmid DNA.

The presence of PTY75-LAC4 in the yeast cells could be proved by means of the Southern hybridization method with ^{32}P-labelled pCR1.

DNA preparations were made according to Struhl et al. (Proc. Natl. Acad. Sci. 76 (1979) 1035-1039).

Example 5

Kluyveromyces lactis SD11 lac4 trp1 transformed to Trp^+ with plasmid pKARS12

Cells of the strain K. lactis SD11 lac4 trp1 were transformed as described in Example 4 with 10 g of pKARS12 DNA. Transformants were selected on 2% agar minimal plates containing 2% of glucose and 0.6M KCl. Per microgram of pKARS12 DNA 3.4×10^4 Trp^+ transformants were obtained.

Example 6

Kluyveromyces lactis SD69 lac4 transformed to Lac^+ with plasmid pL4

K. lactis strain SD69 lac4 was transformed with plasmid pL4 using the same method as described for PTY75-LAC4 in Example 4. The transformants were selected on yeast nitrogen base plates (DIFCO) containing 2% of lactose. The transformation frequence was 20 transformants per microgram of plasmid DNA.

Examples 7-13

Kluyveromyces lactis SD69 lac4 transformed to Trp^+ with KARS-type plasmids.

Analogous to the method described in Example 5, transformation experiments were carried out with other KARS-type plasmids. The results of the experiments are summarized in the following Table.

TABLE

Ex.	Strain	Genotype	Plasmid	Transformants per microgram DNA	Size of KARS fragments (kb)
4.	SD69	lac4	PTY75-LAC4	20	-
7.	SD11	lac4 trp1	pKARS1	1.5×10^3	2.24
8.	SD11	lac4 trp1	pKARS2	5×10^3	1.24
9.	SD11	lac4 trp1	pKARS7	10^3	2.3
10.	SD11	lac4 trp1	pKARS8	5×10^3	1.85
11.	SD11	lac4 trp1	pKARS10	2.4×10^4	3.15
5.	SD11	lac4 trp1	pKARS12	3.4×10^4	5.0
12.	SD11	lac4 trp1	pKARS13	1.5×10^4	2.0
13.	SD11	lac4 trp1	pKARS14	1.8×10^4	2.15

The molecular weights of pKARS plasmids were determined after digestion with endonucleases Eco RI and Hind III, using 0.8% of agarose gel and the usual molecular weight markers.

Example 14

Kluyveromyces lactis SD11 lac4 trp1 transformed to Trp^+ with plasmids containing the KARS-2 sequence using a transformation procedure with whole cells

Plasmid pEK2-7 was used to transform K. lactis SD 11. This plasmid consists of the well-known plasmid YRp7 into which a 1.2 kb fragment containing the autonomously replicating sequence derived from KARS-2 has been cloned (Figure 2). K. lactis SD11 was grown overnight at 30°C in 1% yeast extract, 2% peptone and 2% glucose (pH 5.3). The cells were harvested at OD_{610} nm of 4-8 by centrifugation at 1000xg for 5 minutes. The cells were washed with TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0) and the pellet was resuspended in TE-buffer at a concentration of 2×10^8 cells per ml. This suspension was diluted with one volume of 0.2 M LiCl and shaken at 30°C for 60 minutes.

Plasmid pEK2-7 DNA (10 µg) was added to 0.1 ml of Li-treated cells and the incubation was continued at 30°C for 30 min. One volume of 70% polyethylene glycol 7000 was added and the mixture was incubated for another 60 minutes at 30°C. The mixture was exposed to heat treatment at 42°C for 5 minutes and the cells were washed with sterile, demineralized water. Cells were plated onto minimal agar containing 2% glucose and 0.67% yeast nitrogen base.

Transformants were observed after 36-48 hours at 30°C.

Example 15

Kluyveromyces fragilis transformed with plasmids containing the KARS-2 sequence

Two types of plasmids were used to transform K. fragilis. The first plasmid pGB 180 was constructed by cloning the 3.5 kb Bgl II fragment from plasmid pEK2-7 (Figure 2) containing the KARS-2 autonomously replicating sequence from K. lactis and the TRP1 gene from S. cerevisiae into the Bam Hl site of pJDB 207 (J.D. Beggs, Alfred Benzon Symposium 16 (1981) 383).

About 36 K. fragilis leu mutants obtained after UV-treatment of K. fragilis were transformed with pGB by the Li^+method as described in Example 14. One mutant, K. fragilis leu 24, was transformed to Leu^+ with a frequency of about 10^3 transformants per µg of plasmid DNA.

The second plasmid, pGB 181, was constructed by cloning the 3.5 kb BglII fragment from pEK2-7 as described above into the Bam Hl site of the well-known plasmid pACYC184 which contains the transposon Tn601 conferring resistance to kanamycin and the gentamycin derivative G418.

K. fragilis strain C12 was transformed with plasmid pGB 181 by the Li^+ method as described in Example 14. The transformed cells were plated onto YNPD-agar containing 50 µg of G418 per ml. Transformants were detected after incubation at 30°C for 48 hours, whereas spontaneous resistant mutants were detected only after 6 days. DNA was extracted from K. fragilis transformants and transformed into suitable E. coli DG 75 cells. DNA extracted from kanamycin-resistant E. coli cells showed the presence of plasmid pGB 181.

These experiments show that K. fragilis strains can be transformed by plasmids containing KARS-sequences and that these plasmids are autonomously replicating in K. fragilis.

Example 16

Kluyveromyces lactis SD11 lac4 trp1 expressing preprochymosin and its various maturation forms after being transformed with plasmids containing the KARS-2 sequence, the structural genes encoding preprochymosin and its various maturation forms, and various promotors directing the syntheses of said structural genes.

This Example comprises a number of steps the most essential of which are:

1. Addition of Sal I linkers in front of the cloned structural genes encoding preprochymosin, prochymosin, pseudochymosin and chymosin.
2. Introduction of a DNA fragment in plasmids obtained above containing the KARS-2 autonomously replicating sequence

from K. lactis and the TRP1 gene from S. cerevisiae.

3. Introduction of various promotors into the plasmids obtained above directing the synthesis of the various maturation forms of preprochymosin.

Starting materials for the expression of bovine preprochymosin and its various maturation forms in K. lactis were the following cloned structural genes

- methionyl-pseudochymosin, described as pUR 1531
- methionyl-chymosin , described as pUR 1522
- methionyl-prochymosin , described as pUR 1523
- methionyl-preprochymosin, described as pUR 1524

The construction and structure of these plasmids have been described in detail in European Patent Application No. 82201272.0, published on April 20, 1983 under No. 0077109. The genes were isolated and these plasmids constructed according to the said description.

A. Introduction of Sal I linkers in plasmids pUR 1531, pUR 1522, pUR 1523 and pUR 1524 (Figure 1)

The plasmids pUR 1531, pUR 1522, pUR 1523 and pUR 1524 contain an Eco R1 restriction site just in front of the ATG initiation codon. Because an additional Eco R1 site is present within the chymosin gene, it was aimed to introduce a Sal I linker molecule just in front of the first Eco R1 site to facilitate the introduction of various promotor sequences directing the expression of the distal structural genes.

About 50 µg of DNA was incubated with 50 units of endonuclease Eco R1 in the presence of 125 µg/ml ethidiumbromide in 10 mM Tris-HCl, 50 mM NaCl, 6 mM beta-mercaptoethanol, 10mM $MgCl_2$ and 100 ug/ml bovine serum albumin, pH 7.5, at 37°C for 60 minutes. Plasmid DNA was predominantly converted to linear and open circular molecules under these conditions. The DNA was extracted with one volume of phenol and one

volume of chloroform and precipitated with one volume of propanol-2.
The DNA was dissolved in TE-buffer and completely digested with endonuclease Sal I. A DNA fragment of about 1800 bp was isolated from agarose gel by electroelution.

The fragments were extracted with phenol and chloroform and precipitated with propanol-2. The precipitates were dissolved in TE-buffer.
The cohesive ends were filled-in with DNA polymerase as follows:
To 15 µl containing the 1800 bp DNA fragment (about 1-2 µg) was added 1 µl of a 2 mM solution of dATP, dGTP, dCTP and dTTP, 6.5 µl of 4x nick-buffer containing 0.2 M Tris-HCl (pH 7.2), 40 mM $MgSO_4$, 4 mM dithiothreitol and 200 mg/ml bovine serum albumin, and 2.5 µl of water. Two units of DNA polymerase (Klenow fragment) were added and the mixture was incubated at 20°C for 30 minutes. DNA polymerase was then inactivated by heating at 70°C for 5 minutes.
A phosphorylated Sal I-linker (prepared as described in Maniatis et al, Molecular Cloning, CSH) was added to this mixture together with T4 DNA ligase (10^3 Units).
After incubation at 22°C for 4 hours the mixture was incubated at 4°C for an additional 16 hours. The mixture was then incubated with endonucleases Sal I and Hind III and a DNA fragment of about 1500 bp was recovered from an agarose gel by electroelution.
The fragments (A,B,C,D) were purified by phenol and chloroform extraction and precipitation with propanol-2.
These fragments were ligated to a 3.3 kb Hind III-Sal I fragment (about 0.5 µg) derived from plasmid pPA153-209 containing a temperature-sensitive replicon and an ampicillin resistant gene (encoding beta-lactamase), and purified from an agarose gel by electroelution.
The ligated molecules were transformed into E. coli HB 101 and ampicillin resistant, tetracyclin sensitive clones were cultured and plasmid DNA extracted. Digestion of plasmid DNA with enconucleases Sal I, Eco Rl and Hind III confirmed that

the plasmids pGB131, pGB122, pGB123 and pGB124 (Figure 1) were obtained.

B. Introduction of a KARS2 and TRP1 gene in the plasmids pGB131, pGB122, pGB123 and pGB124, respectively.

Autonomously replicating sequences derived from and replicating in Kluyveromyces were obtained as described in Examples 2 and 7-15. The autonomously replicating sequence in plasmid pKARS-2 is located on a 1.24 kb fragment and this fragment was cloned into the well-known plasmid YRp7 and a new plasmid pEK2-7 was obtained (Figure 2). Digestion of pEK2-7 with endonuclease Cla I resulted in fragments of 3.5 and 5.5 kb, respectively. The 3.5 kb fragment containing the TRP1 gene derived from S. cerevisiae and the KARS-2 sequence derived from K. lactis (Figure 2) was isolated from an agarose gel by electroelution and ligated to Cla I-digested plasmids pGB131, pGB122, pGB123 and pGB124, respectively. The resulting mixture was transformed into E. coli JA300 (trpC) and characterization of plasmid DNA extracted from Trp^+ transformants confirmed the construction of plasmids pGB151, pGB152, pGB153 and pGB154, respectively (Figure 2).

C. Introduction of various promotor sequences in the plasmids directing the synthesis of the various maturation forms of preprochymosin.

The Sal I-digested plasmids containing the KARS-2 sequence, the TRP1 gene and the structural gene of preprochymosin or its various maturation forms are well suited to accept Sal I-linked promotor sequences to direct the synthesis of the distal structural gene in K. lactis transformants.
In most cases the promotor sequences have to be provided with Sal I linkers. Any promotor sequence can be provided with such a Sal I linker and in the following Examples this is illustrated with

1. the isocytochrome c1 promotor from S. cerevisiae
2. the lactase promotor from K. lactis

C1. Addition of Sal I linkers to the isocytochrome c1 promotor from S. cerevisiae and introduction into plasmids. (Figure 3)

Plasmid pYeCYC1 consisting of the isocytochrome c1 gene cloned into plasmid pBR322 was used as the starting material (G. Faye et al., Proc. Natl. Acad. Sci. USA 78 (1981) 2258).

From nucleotide sequence data it is known that an Eco RI site is present in the isocytochrome c1 gene at nucleotide +8 (Ibid.)

Plasmid pYeCYC1 was cleaved with endonuclease Eco R1, ligated with T4 DNA ligase and transformed into E. coli HB101, yielding a plasmid pC15 containing the 1930 bp fragment carrying the promotor and 8 nucleotides of the isocytochrome c1 gene.

Plasmid pC15 was cleaved with endonuclease Eco RI and incubated with nuclease Bal 31 for a short while to remove just a few nucleotides.
The Bal 31 digested ends were converted to blunt-ends with DNA polymerase (Klenow fragment) and a phosphorylated Eco RI linker was ligated to this DNA. After incubation with endonuclease Eco RI, ligation and transformation into E. coli, a transformant pC15-R12 was indentified in which 12 nucleotides from the cytochrome c1 gene had been removed.
A Sal I linker was introduced by cleaving plasmid pC15-R12 with endonuclease Eco RI, filling in the cohesive ends with DNA polymerase, ligation of a phosphorylated Sal I linker, incubation with endonuclease Sal I and recloning the resulting 1070 bp fragment in the Sal I digested plasmids pGB151, pGB152, pGB153 and pGB154, respectively, yielding the isocytochrome c1 promotor containing plasmids pGB161, pGB162, pGB163 and pGB164, respectively as identified by colony hybridization with the ^{32}P-labeled 1070 bp fragment as probe.
Plasmid DNA was prepared from the positive clones and the

correct orientation of the isocytochrome c1 promotor was confirmed by the presence of a 850 bp fragment after digestion with endonuclease Sma I.

C2. Addition of Sal I linkers to the lactase promotor from Kluyveromyces lactis and introduction into plasmids.

The starting material was plasmid pK16 containing the lactase gene from K. lactis cloned into the Eco RI site of plasmid pBR322 (R.C. Dickson and J.S. Markin, Cell 15 (1978), 123).
Sequencing of large parts of the lactase structural gene and its promotor established the presence of a Cla I site at about 450 bp in the lactase structural gene.
Plasmid pK16 was digested with endonuclease Cla I and the fragment containing the promotor and about 450 bp of the structural gene were recloned into the plasmid pBR322 digested with endonucleases Cla I and Acc I (partially).
In one plasmid, pGB 182, the retained Cla I site at about 450 bp in the lactase structural gene was opened by incubation with endonuclease Cla I and trimmed by incubation with nuclease BAl 31. The Bal 31 ends were rendered blunt-ends by incubation with DNA polymerase and a phosphorylated Eco RI linker was ligated to this trimmed fragment.
Digestion with endonuclease Eco RI and recloning of the trimmed fragment resulted in plasmid pGB 183, that had retained the lactase promotor and was devoid of the structural gene.
Sal I linkers were added to this fragment as described in the previous example (16.C2). The Sal I linked lactase promotor was ligated to Sal I-cleaved plasmids pGB 151, pGB 152, pGB 153 and pGB 154, respectively, yielding plasmids pGB 171, pGB 172, pGB 173 and pGB 174, respectively.

Plasmids obtained as described in this Example 16 were introduced into Kluyveromyces lactis SD11 lac4 trp1 by the Li^+ method as described in Example 14, selecting for Trp^+ transformants.

The presence of preprochymosin or its maturation forms in Kluyveromyces extracts was demonstrated by immunological ELISA techniques and by spotting aliquots of the extracts on nitrocellulose filters and assaying the filters as described by D.J. Kemp and A.F. Cowman (Proc. Natl. Acad. Sci. USA 78 (1981) 4520-4524).

Cell-extracts were prepared as follows: K. lactis transformants were grown at 30°C for about 16-24 hours in YNB-medium containing 2% dextrose.
Cells were harvested at OD_{610nm} between 2.2-6.0 by centrifugation at 6000 pm for 10 minutes in a Sorvall G-S3 rotor. The pellet was resuspended in sterile destilled water to OD_{600} of 600 and chilled on ice.

0.5 ml of this cell suspension was diluted with 0.5 ml of ice-cold water and mixed with 2 g of Ballotini beads (diameter 0.25-0.35 mm Braun-Melsungen GMBH, GFR).

The cells were disrupted by shaking for 4 minutes on a Vortex shaker at maximal speed.
More than 95% of the cells were disrupted as checked by phase contrast microscopy. Cell debris was removed by centrifugation for 1 minute in an Eppendorf centrifuge.
Aliquots of the extracts were frozen in liquid nitrogen and stored at -80°C.

1-5 µl aliquots of the cell extracts were spotted on nitrocellulose membrane filters. The filters were dried, wetted with 192 mM glycine, 25 mM Tris, 20% ethanol (pH 8.3) and incubated for 60 minutes at 22°C.
The filters were subsequently incubated with preincubation buffer (0.35M NaCl, 10 mM Tris-HCl (pH 7.6), 2% bovine serum albumin) for 30 minutes. The filters were washed three times for 5 minutes with RIA-buffer (0.125M NaCl, 10 mM Tris-HCl, pH 7.6, 0.1 mM PMSF, 1% Triton X100, 0.5% sodium desoxycholatae, 0.1% sodium dodecylsulfate and 0.3% gelatin).
The filters were incubated overnight at 4°C in 1 ml RIA

buffer containing 10 µl of chymosin antiserum. Antiserum was removed by washing with RIA buffer (three times) and incubated with 1 µCi ^{125}I-protein A in 1 ml of RIA-buffer for 60 minutes at 22°C.

^{125}I-protein A was removed by washing with RIA buffer (5 times).

The filters were dried and autoratiographed overnight.

The presence of preprochymosin or its maturation forms in K. lactis transformantas was clearly observed.

The presence of chymosin activity in cell extracts from K. lactis transformants was determined by high performance liquid chromatrography (HPLC) as described by A.C.M. Hooydonk and C. Olieman, Netherl. Milk Dairy 36 (1982), 153.

50 µl of enzyme solution or extract was added to 1 ml of a 10% solution of milkpowder (Difco) in 10 mM $CaCl_2$.

The solution was incubated for 15 minutes at 31°C.

The reaction was stopped by adding 2 ml of 12% trichloroacetic acid (TCA). Almost all proteins are precipitated by TCA except glycomacropeptide (GMP) that has been cleaved from κ casein by chymosin action.

Denatured proteins are pelleted by centrifugation and 1 ml of the clear supernatant was neutralised with 0.4 ml of 1N NaOH.

The solution was centrifuged again and the amount of GMP produced was detected with HPLC monitoring the extinction at 214 nm.

Extracts from K. lactis transformants containing prochymosin were first incubated at pH 2 for 2 hours and subsequently neutralized before performing the chymosin activity test. Chymosin was only found after the pH 2 treatment.

Example 17

Kluyveromyces SD11 lac4 trp1 expressing preprothaumatin and its various maturation forms after being transformed with plasmid pURK 528-01 containing the structural gene encoding preprothaumatin, the KARS2 sequence from K. lactis, the glyceraldehyde-3-phosphate dehydrogenase promotor from S. cerevisiae and the TRP1 gene from S. cerevisiae

This Example comprises a number of steps the most essential of which are:

1. Isolation of clones containing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) operon of S. cerivisiae

A DNA pool of the yeast S. cerevisiae was prepared in the hybrid E. coli-yeast plasmid pF1 (M.R. Chevallier et al., Gene 11 (1980) 11-19) by a method similar to the one described by M. Carlson and D. Botstein, Cell 28 (1982) 145-154. Purified yeast DNA was partially digested with restriction endonuclease Sau 3A and the resulting DNA fragments (with an average length of 5 kb) were ligated by T4 DNA ligase in the dephosphorylated Bam HI site of pF1 1. After transformation of $CaCl_2$-treated E. coli cells with the ligated material a pool about 30.000 ampicillin resistant clones was obtained. These clones were screened by a colony hybridization procedure (R.E. Thayer, Anal. Biochem., 98 (1979) 60-63) with a chemically synthesized and ^{32}P-labeled oligomer with the sequence 5'TACCAGGAGACCAACTT3'.

According to data published by J.P. Holland and M.J. Holland (J. Biol. Chem., 255 (1980) 2596-2605) this oligomer is complementary with the DNA sequence encoding aminoacids 306-310 (the wobble base of the last amino acid was omitted from the oligomer) of the GAPDH gene. Using hybridization conditions described by R.B. Wallace et al., Nucleic Acid Res., 9 (1981) 879-894, six positive transformants could be identified. One of these harboured plasmid pF1 1-33. The latter plasmid contained the GAPDH gene including its

promotor/regulation region and its transcription termination/polyadenylation region. The approximately 9 kb long insert of pF1 1-33 has been characterized by restriction enzyme analyses (Fig. 4) and partial nucleotide sequence analysis (Figs. 5 and 6).

2. Isolation of the GAPDH promotor/regulation region and its introduction into a preprothaumatin encoding plasmid

On the basis of the restriction enzyme analysis and the nucleotide sequence data of the insert of plasmid pF1 1-33, the DNA initiation/regulation region of the GAPDH gene was isolated as an 800 nucleotides long Dde I fragment. To identify this promotor fragment, plasmid pF1 1-33 was digested with Sal I and the three resulting DNA fragments were subjected to a Southern blot hybridization test with the chemically synthesized oligomer (E.M. Southern, J. Mol. Biol. 98 (1975) 503-517).

A positively hybridizing 4.3 kb long restriction fragment was isolated on a preparative scale by electroelution from a 0.7% agarose gel and was then cleaved with Dde I. Of the resulting Dde I fragments only the largest one had a recognition site for Pvu II, a cleavage site located within the GADPH regulon region (Fig. 1). The largest Dde I fragment was isolated and incubated with Klenow DNA polymerase and four dNTP's (A.R. Davis et al., Gene 10 (1980) 205-218) to generate a blunt-ended DNA molecule. After extraction of the reaction mixture with phenol/chloroform (50/50 v/v), passage of the aqueous layer through a Sephadex G50 column and ethanol precipitation of the material present in the void volume, the DNA fragment was equipped with the ^{32}P-labeled Eco RI linker 5'GGAATTCC3' by incubation with T4 DNA ligase. Due to the Klenow polymerase reaction and the subsequent ligation of the Eco RI linker, the original Dde I sites were reconstructed at the end of the regulon fragment. To inactivate the ligase the reaction mixture was heated to 65°C for 10 minutes, then sodium chloride was added (final concentration 50 mmol/l) and the whole mix was incubated

with Eco RI. Incubation was terminated by extraction with phenol/chloroform, the DNA was precipitated twice with ethanol, resuspended and then ligated into a suitable vector molecule. Since the Dde I regulon fragment was equiped with Eco RI sites it can be easily introduced into the Eco RI site of pUR 528 (EP-PA 54331) to create a plasmid in which the yeast regulon is adjacent to the structural gene encoding preprothaumatin. The latter plasmid was obtained by cleavage of pUR 528 with Eco RI, treatment of the linearized plasmid molecule with (calf intestinal) phosphatase to prevent self-ligation and incubation of each of these vector molecules as well as the purified Dde I promotor fragment with T4 DNA ligase. Transformation of the various ligation mixes in $CaCl_2$-treated E. coli HB101 cells yielded several ampicillin resistant colonies. From some of these colonies plasmid DNA was isolated (H.C. Birnboim and J. Doly, Nucleic Acids Res. 7 (1979) 1513-1523) and incubated with PvuII to test the orientation of the insert.

In the nomenclature plasmids containing the Eco RI (Dde I) GAPDH regulon fragment in the correct orientation (i.e. transcription from the GAPDH regulon occurs in the direction of a downstream located structural gene) are indicated by the addendum-01 to the original code of the plasmid (for example pUR 528 is changed in pUR 528-01; see Fig. 7).

To facilitate manipulation of plasmids containing the Eco RI regulon fragment, one of the two Eco RI sites was destroyed. Two µg of plasmid DNA (e.g. pUR 528-01) was partially digested with Eco RI and then incubated with 5 units Mung bean nuclease (obtained from P.L. Biochemicals Inc.) in a total volume of 200 µl in the presence of 0.05 moles/l sodium acetate (pH 5.0), 0.05 moles/l sodium chloride and 0.001 moles/l zinc chloride for 30 minutes at room temperature to remove sticky ends. The nuclease was inactivated by addition of SDS to a final concentration of 0.1% (D. Kowalski et al., Biochemistry 15 (1976) 4457-4463 and the DNA was precipitated by the addition of 2 volumes of ethanol

(in this case the addition of 0.1 volume of 3 moles/l NaAc was omitted. Linearized DNA molecules were then religated by incubation with T4 DNA ligase and used to transform $CaCl_2$-treated E. coli cells. Plasmid DNA isolated from ampicillin resistant colonies was tested by cleavage with Eco RI and Mlu I for the presence of a single Eco Rl site adjacent to the thaumatin gene (cf. Fig. 7).

Plasmids containing the GAPDH promotor fragment but having only a single Eco RI recognition site adjacent to the ATG initiation codon of a downstream located structural gene are referred to as -02 type plasmids (for example: pUR 528-01 is changed in pUR 528-02; see Fig. 7).

3. Reconstitution of the original GAPDH promotor/regulation region in plasmids encoding preprothaumatin by introduction of a synthetic DNA fragment (Fig. 8)

As shown by the nucleotide sequence depicted in Fig. 5, the Eco RI (Dde I) GAPDH promotor fragment contains the nucleotides -850 to -39 of the original GAPDH promoter/regulation region. Not contained in this promoter fragment are the 38 nucleotides preceding the ATG initiation codon of the GAPDH encoding gene. The latter 38-nucleotides long fragment contains the PuCACACA sequence, which is found in several yeast genes. Said PuCACACA sequence situated about 20 bp upstream of the translation start site (M.J. Dobson et al., Nucleic Acid Res., 10 (1982) 2625-2637) provides the nucleotide sequence upstream of the ATG codon which is optimal for protein initiation (M. Kozak, Nucleic Acids Res. 9 (1981) 5233-5252). Moreover, these nucleotides allow the formation of a small loop structure which might be involved in the regulation of expression of the GAPDH gene.
On the basis of the above-mentioned arguments, introduction of the 38 nucleotides in between the Dde I promotor-fragment and the ATG codon of a downstream located structural gene was considered necessary to improve promotor activity as well as translation initiation.

As outlined in Fig. 9 the missing DNA fragment was obtained by the chemical synthesis of two partially overlapping oligomers. The Sac I site present in the overlapping part of the two oligonucleotides was introduced for two reasons: (i) to enable manipulation of the nucleotide sequence immediately upstream of the ATG codon including the construction of poly A-tailed yeast expression vectors; (ii) to give a cleavage site for an enzyme generating 3'-protruding ends that can easily and reproducibly be removed by incubation with T4 DNA polymerase in the presence of the four dNTP's. Equimolar amounts of the two purified oligomers were phosphorylated at their 5'-termini, hybridized (J.J. Rossi et al., J. Biol. Chem. 257 (1982) 9226-9229) and converted into a double-stranded DNA molecule by incubation with Klenow DNA polymerase and the four dNTP's under conditions which have been described for double-stranded DNA synthesis (A.R. Davis et al., Gene 10 (1980) 205-218). Analysis of the reaction products by electrophoresis through a 13% acrylamide gel followed by autoradiography showed that more than 80% of the starting single-stranded oligonucleotides were converted into double-stranded material. The DNA was isolated by passage of the reaction mix over a Sephadex G50 column and ethanol precipitation of the material present in the void volume. The DNA was then phosphorylated by incubation with polynucleotide kinase and digested with Dde I. To remove the nucleotides cleaved off in the latter reaction, the reaction mix was subjected to two precipitations with ethanol.

As shown in Fig. 8 cloning of the resulting synthetic DNA fragment was carried out by the simultaneous ligation of this fragment and a BglII-DdeI GAPDH promoter regulation fragment in a vector molecule from which the Eco RI site preceding the ATG initiation codon was removed by Mung bean nuclease digestion (cf. E.). The BglII-DdeI promoter/regulation fragment was obtained by digestion of plasmid pUR 528-02 with DdeI and BglII. Separation of the resulting restriction fragments by electrophoresis through a 2%

agarose gel and subsequent isolation of the fragment from the gel yielded the purified 793 nucleotides long promotor/regulation fragment. In the plasmid pUR 528-02 the nucleotide sequence preceding the ATG codon is 5'-GAATTC(T)ATG-3' (EP-PA 54330 and EP-PA 54331), which is different from the favourable nucleotide sequence given by M. Kozak (Nucleic Acids Res. 9 (1981) 5233-5252). Since our aim was to reconstitute the original GAPDH promotor/regulation/protein initiation region as accurately as possible, the Eco RI site was removed in order to ligate the synthetic DNA fragment to the resulting blunt-end. Removal of the Eco RI site was accomplished by Mung bean nuclease digestion of Eco RI-cleaved pUR 528-02 DNA.

Subsequently the plasmid DNA was digested with BglII and incubated with phosphatase. After separation of the two DNA fragments by electrophoresis through a 0.7% agarose gel, the largest fragment was isolated and used as the vector in which the BglII-DdeI promoter fragment as well as the -DdeI-treated - synthetic DNA fragment were ligated.
Plasmids in which the DdeI promotor/regulation fragment together with the Sac I recognition site containing synthetic DNA fragment are introduced are indicated by the addendum -03 (for example: pUR 528-02 is changed into pUR 528-03).

4. Introduction of the KARS2 replicon from K. lactis and the TRP1 gene from S. cerevisiae in preprothaumatin encoding plasmids

The KARS2 replicon and the TRP1 gene were excised from pEK 2-7 by digestion with Bgl II, followed by isolation from a 0.7% agarose gel of the 3.5 kb fragment. This purified fragment was inserted in the dephosphorylated Bgl II cleavage site of pUR 528-03 by incubation with T4 DNA ligase. Transformation of the ligation mix in E. coli yielded plasmid pURK 528-03 (Fig. 10). Transformants generated by the introduction of plasmid pURK 528-03 into K. lactis SD11 cells by the Li^+ method were shown to synthesize

thaumatin-like proteins assayed as described by L. Edens et al., Gene 18 (1982) 1-12, see Fig. 11.

CLAIMS

1. A yeast cell of the genus Kluyveromyces as a host capable of expressing an inserted polypeptide coding sequence.

2. A yeast cell of the genus Kluyveromyces as a host capable of expressing an inserted polypeptide coding sequence derived from recombinant DNA material.

3. A yeast cell according to claim 2, wherein the inserted sequence encodes chymosin.

4. A yeast cell according to claim 2, wherein the inserted sequence encodes prochymosin.

5. A yeast cell according to claim 2, wherein the inserted sequence encodes preprochymosin.

6. A yeast cell according to claim 2, wherein the inserted sequence encodes pseudochymosin.

7. A yeast cell according to claim 2, wherein the inserted sequence encodes beta-galactosidase.

8. A yeast cell according to claim 2, wherein the recombinant DNA material comprises

(i) a polypeptide coding sequence,

(ii) a vector in which the polypeptide coding sequence is inserted,

(iii) a regulon directing the expression of the polypeptide,

and optionally

(iv) a selection marker,

(v) a transcription terminator,

(vi) a sequence allowing the vector to replicate, and

(vii) a centromer.

9. A yeast expression vector comprising

(i) a polypeptide coding sequence,

(ii) a regulon directing the expression of the polypeptide,

and optionally

(iii) a selection marker,

(iv) a transcription terminator,

(v) a sequence allowing the vector to replicate, and

(vi) a centromer.

10. A yeast expression vector comprising a selective marker, a replication origin and a promotor directing the expression of a downstream polypeptide encoding sequence, the yeast replication containing at least a portion of an autonomous replicating sequence originating from Kluyveromyces.

11. A yeast expression vector comprising a selective marker, a promotor and homologous Kluyveromyces DNA acting as a site for recombination with the host chromosome.

12. A process for preparing a new strain of the yeast Kluyveromyces, which comprises

(i) transforming yeast cells of the genus Kluyveromyces by a vector containing a polypeptide coding sequence which can be expressed in the host cells,

(ii) allowing the yeast cells to grow under a selection pressure.

13. A process according to claim 12, wherein the transformation is carried out with protoplasts.

14. A process according to claim 12, wherein the transformation is carried out with whole cells.

15. A process according to claim 12, wherein the vector contains one or more sequences which control the function of replication and maintenance within the Kluyveromyces cells.

16. A process according to claim 12, wherein the control sequences are selected from the group of autonomously replicating sequences (ARS).

17. A process according to claim 16, wherein the control sequence is an autonomously replicating sequence originating from Kluyveromyces (KARS).

18. A process according to claim 17, wherein the control sequence is selected from the group consisting of the KARS12 and KARS2 sequence.

19. A process according to claim 12, wherein the vector contains homologous Kluyveromyces DNA acting as a site for recombination with the host chromosome.

20. A process according to claim 12, wherein the vector is selected from the group consisting of pKARS12, pKARS2, pL4 and PTY75-LAC4.

21. A process according to claims 12-20, wherein the vector contains a centromer region originating from Kluyveromyces or Saccharomyces chromosomes.

22. A process according to claims 12-21, wherein the transformed genes are integrated in the chromosomal DNA of the host.

23. A process according to claims 12-22, wherein the transformed yeast cells are incubated in a medium containing potassium chloride.

21. A process according to claim 23, wherein the concentration of potassium chloride is about 0.6M.

22. A process according to claims 12-21, wherein the polypeptide coding sequence is selected from the group consisting of kanamycin resistance, beta-galactosidase,

amyloglucosidase, alpha-amylase, invertase, beta-lactamase, chymosin and its precursors, TRP1 and LEU2.

23. A process according to one or more of the preceding claims, wherein the Kluyveromyces cells originate from Kluyveromyces lactis.

24. A process according to one or more of the preceding claims, wherein the Kluyveromyces cells originate from Kluyveromyces fragilis.

25. A process according to one or more of the preceding claims, wherein the Kluyveromyces cells originate from K. lactis SD11 lac4 trp1 or K. lactis SD69 lac4.

26. Kluyveromyces cells transformed by a vector containing the 2 micron sequence from Saccharomyces or an autonomously replicating sequence from Saccharomyces or Kluyveromyces.

27. Kluyveromyces cells transformed by a vector containing an autonomously replicating sequence from Kluyveromyces lactis.

28. Kluyveromyces cells transformed by a vector which is wholly or partially constituted by one of the plasmids pKARS12, pKARS2, pL4 or PTY75-LAC4 or by parts thereof.

29. Kluyveromyces lactis SD69 lac4 (PTY75-LAC4).

30. Kluyveromyces lactis SD11 lac4 trp1 (pKARS12).

31. Kluyveromyces lactis SD69 lac4 (pL4).

32. Vectors containing as a constituent a centromer region originating from Kluyveromyces or Saccharomyces.

33. Plasmid pKARS12.

34. Plasmid pKARS2.

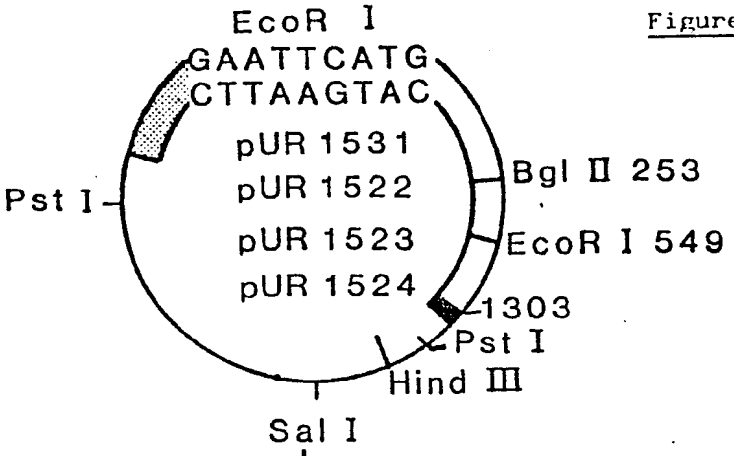
35. Plasmid pL4.

36. Plasmid PTY75-LAC4.

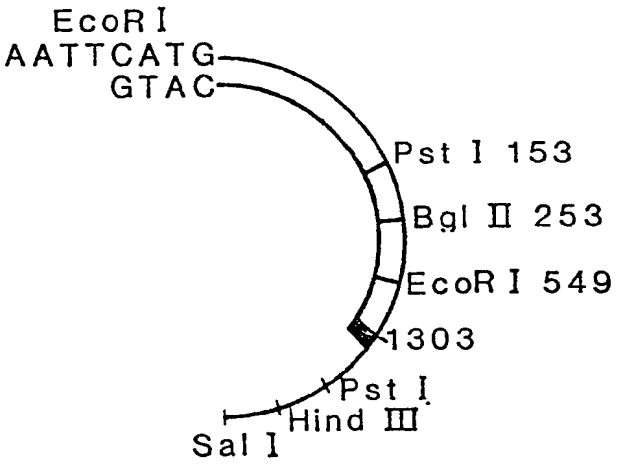
37. A process of preparing polypeptides which comprises cultivating a yeast of the genus Kluyveromyces as claimed in one or more of claims 1-8.

38. Polypeptides prepared according to claim 37.

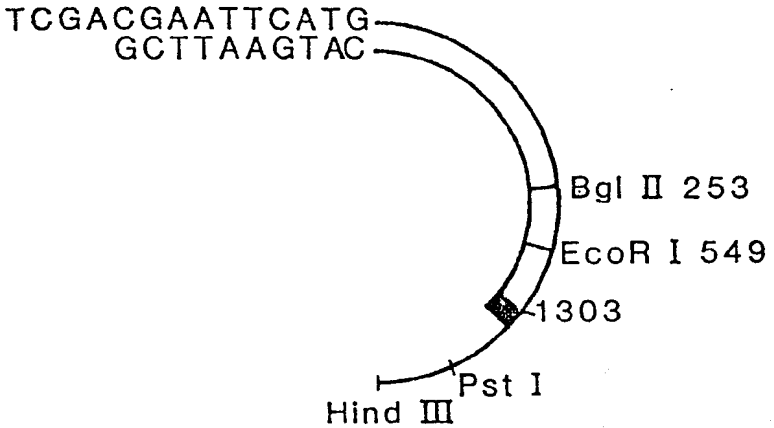
Figure 1



1. Partial EcoR I digestion (in the presence of ethidium bromide)
2. Sal I digestion
3. Purify EcoR I-Sal I fragments (1900-2150 bp) from agarose gel

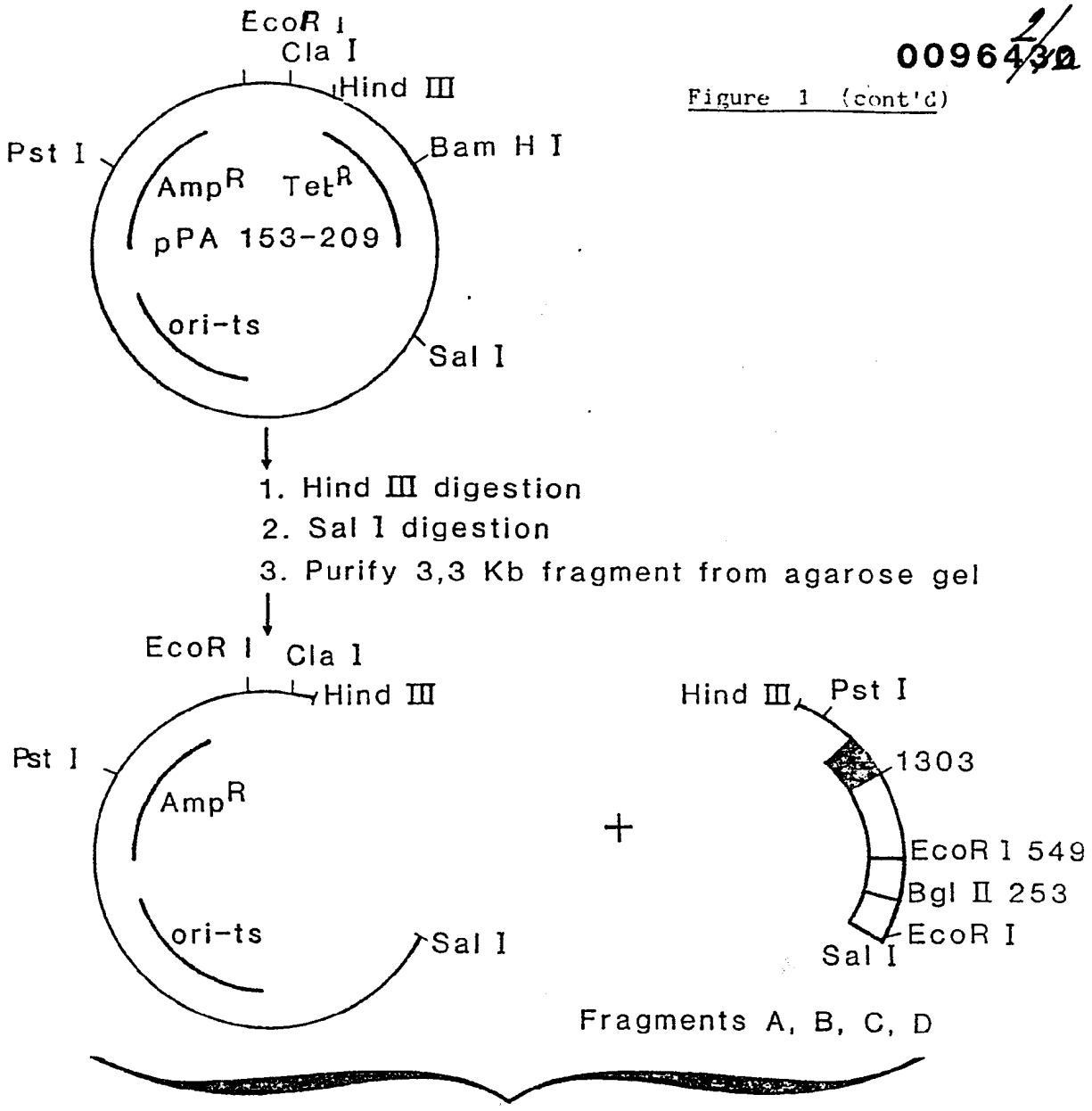


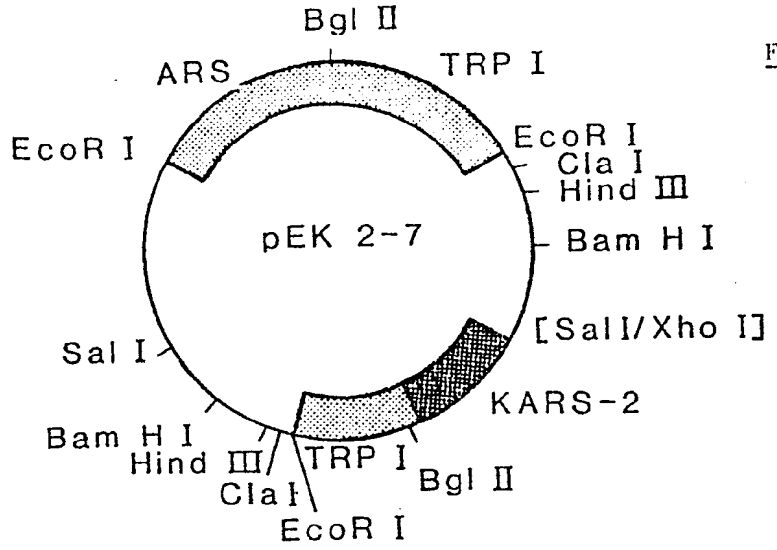
1) Fill-in cohesive ends with DNA polymerase (Klenow-fragment),4 dNTP's
2) Add Sal I - linker (CGTCGACG / GCAGCTGC) with T4 DNA ligase,ATP
3) Hind III digestion
4) Sal I digestion
5) Purify Sal I - Hind III fragments from agarose gel



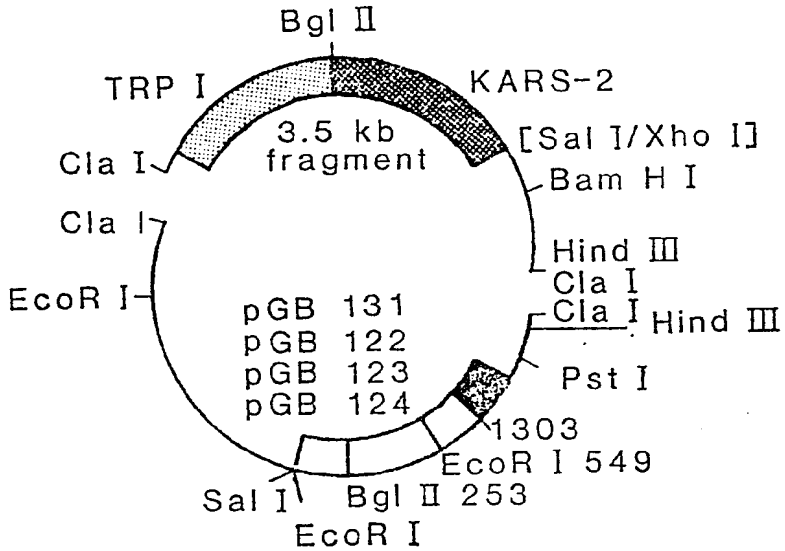
Fragments A, B, C, D

Figure 1 (cont'd)





1. Cla I digestion
2. Purify 3.5 kb fragment from agarose gel
3. Mix with Cla I-digested pGB 131
   pGB 122
   pGB 123
   pGB 124



1. Ligation with T4 ligase, ATP
2. Transformation of E coli JA 300
3. Selection for Trp+ -transformants

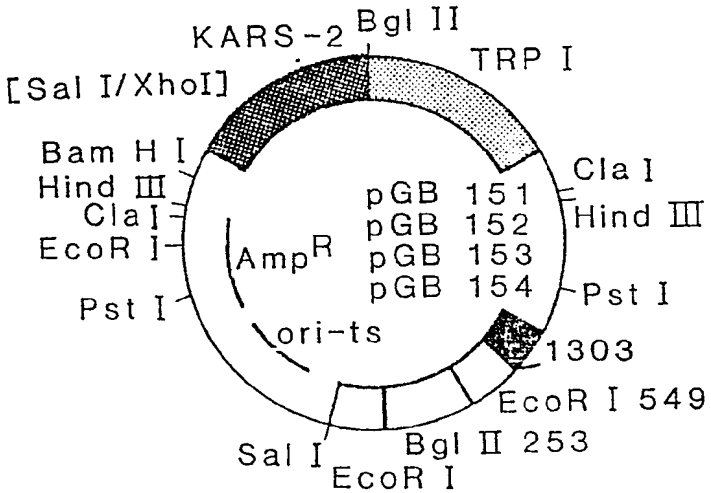
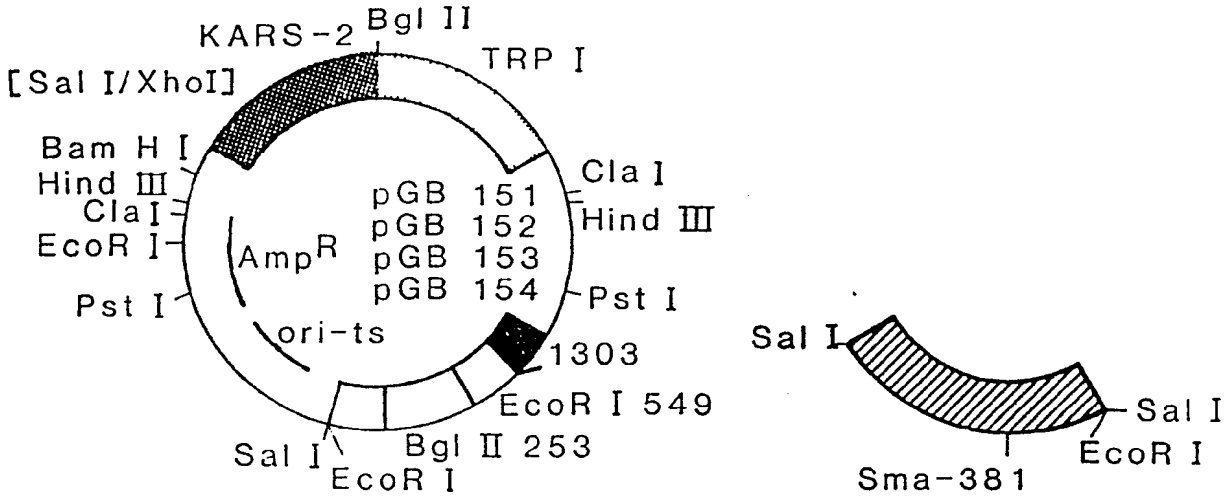


Figure 3



Sal I digestion

cytochrome c1
promotor fragment

1. ligation with T4 DNA ligase
2. transformation of E. coli HB 101

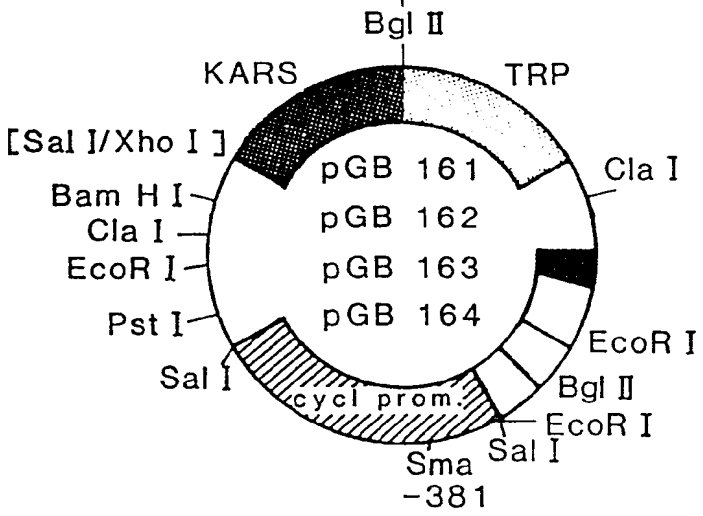
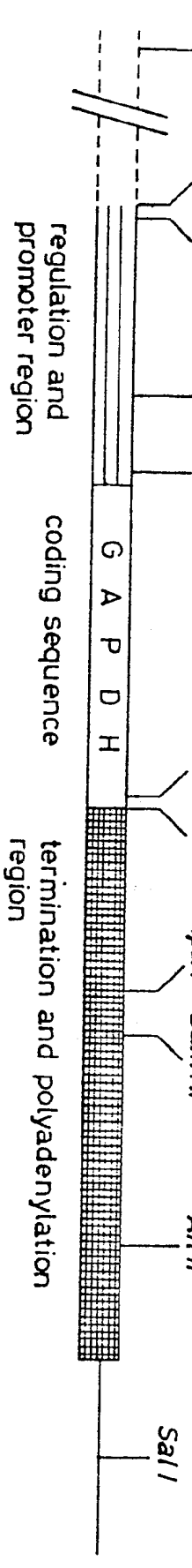


Figure 4


Sal I
Dde I
Bgl II
Pvu II
Dde I
regulation and promoter region
G A P D H
coding sequence
Sal I
Afl II
Hpa I
Bam HI
Afl II
Sal I
termination and polyadenylation region

```
      -840        -830        -820        -810        -800        -790
GAATTCCTCA GTTTCAAGAT CTTTTAATGT CCAAAACCAT TTGAGCCGAT CTAAATACTT
      -780        -770        -760        -750        -740        -730
CTGTGTTTTC ATTAATTTAT AAATTGTACT CTTTTAAGAC ATGGAAAGTA CCAACATCGG
      -720        -710        -700        -690        -680        -670
TTGAAACAGT TTTTCATTTA CATATGGTTT ATTGGTTTTT CCAGTGAATG ATTATTTGTC
      -660        -650        -640        -630        -620        -610
GTTACCCTTT CGTAAAACTT CAAACACGTT TTTAAGTATT GTTTAGTTGC TCTTTCGACA
      -600        -590        -580        -570        -560        -550
TATATGATTA TCCCTGCGCG GCTAAAGTTA AAGATGCAAA AAACAGAAGA CAACTGAAGT
      -540        -530        -520        -510        -500        -490
TAATTTACGT CAATTAAGTT TTCCAGGGTA ATGATGTTTT GGGCTTCCAC TAATTCAATA
      -480        -470        -460        -450        -440        -430
AGTATGTCAT GAAATACGTT GTGAAGAGCA TCCAGAAATA ATGAAAAGAA ACAACGAAAC
      -420        -410        -400        -390        -380        -370
TGGGTCGGCC TGTTGTTTCT TTTCTTTACC ACGTGATCTG CGGCATTTAC AGGAAGTCGC
      -360        -350        -340        -330        -320        -310
GCGTTTTGCG CAGTTGTTGC AACGCAGCTA CGGCTAACAA AGCCTAGTGG AACTCGACTG
      -300        -290        -280        -270        -260        -250
ATGTGTTAGG GCCTAAAACT GGTGGTGACA GCTGAAGTGA ACTATTCAAT CCAATCATGT
      -240        -230        -220        -210        -200        -190
CATGGCTGTC ACAAAGACCT TGCGGACCGC ACGTACGAAC ACATACGTAT GCTAATATGT
      -180        -170        -160        -150        -140        -130
GTTTTGATAG TACCCAGTGA TCGCAGACCT GCAATTTTTT TGTAGGTTTG GAAGAATATA
      -120        -110        -100         -90         -80         -70
TAAAGGTTGC ACTCATTCAA GATAGTTTTT TTCTTGTGTG TCTATTCATT TTATTATTGT
       -60         -50         -40         -30         -20         -10
TTGTTTAAAT GTTAAAAAAA CCAAGAACTT AGTTTCAAAT TAAATTCATC ACACAAACAA
        -1
ACAAAACAAA ATG
```

Fig. 5

```
         7          17          27          37          47          57
TAAATTTAAC TCCTTAAGGT TACTTTAATG ATTTAGTTTT TATTATTAAT TTAAACTTCT
        67          77          87          97         107         117
CATGACATCT CATATACACG TTTATAAAAC TTAAATAGAT TGAAAATGTA TTAAAGATTC
       127         137         147         157         167         177
CTCAGGGATT CGATTTTTTT GGAAGTTTTT GTTTTTTTTT CCTTGAGATG CTGTAGTATT
       187         197         207         217         227         237
TGGGAACAAT TATACAATCG AAAGATATAT GCTTACATTC GACCGTTTTA GCCGTGATCA
       247         257         267         277         287         297
TTATCCTATA GTAACATAAC CTGAAGTATA ACTGACACTA CTATCATCAA TACTTGTCAC
       307         317         327         337         347         357
ATGAGAACTC TGTGAATAAT TAGGCCACTG AAATTTGATG CCTGAAGGAC CGGCATCACG
       367         377         387         397         407         417
TATCTTCGAT AAAGCACTTA GTATCACACT AATTGGCTTT TCGCCGCATA TGGTGTTTCC
       427         437         447         457         467         477
GGTGATTTCC AAGTATTGTT TCCAAGCATC GTACCTTTCA CCATTTGGAG TATCACTTAG
       487         497         507         517         527         537
CGTTTTCATC GCATATCTGT CCATTATTTC AATGGATTGC CAAATGGGAA CTTGATGATG
       547         557         567         577         587         597
TGAAAGTTTA CTCCTAGCAG TTAACATTTC CACTTCTGTT TCCTCTTTAA TGGCATTCAT
       607         617         627         637         647         657
TCAACTCTTC CTTGCTTACC GACGTACCCG TATATTGGAA TCTGCGGCCC CAATGACAGA
       667         677         687         697         707         710
AATCACTGCT TACAATGAAT AAATTGTTCG GATCCTTAAT GTACTCCGAC AAAATATTAC
       727         737         747         757         767         777
CAATGCAACG ATCAACATCA ACGCTGTTAT GAGAAACCAT CATGGGAATT ACCTTCACCG
       787         797         807         817         827         837
TATCTAAAGA AATTTCTCTC CATTTCAAAG TTTCCACCAA CATGGGGAGC TGCATCTCTA
       847         857         867         877         887         897
AGGAATGTTC AGCCATATCA GTGTCATGAT CCATTGGCTT AAACAGCTTC TTTCCGTTCT
       907         917         927         937         947         957
CAGGATACTC CTTCTGTATT AATGTTTTAC ACAAGTCTGT ATCCACTTTC AGATTACCCA
       967         977         987         997        1007        1017
AGGGCGTCTC TAGCTCACTG AATGCACTAA CTAAAATTTG GTTTTTGAAA TAGATGTGAT
      1027        1037        1047        1057        1067        1077
GCGACGGCCC CAAGATAAAT ATTCTCTTAA CATTACGGTT CAAATCCAAC GATGCGTACG
      1087        1097        1107        1117        1127        1137
AGTAGGCCAT AGTGGGTCCA CAATACCTGT AACCGGCATG AGGACATATG ATAATTCTGG
      1147        1157        1167        1177        1187        1197
CGTTGTGAAT TGGGCCTTTA AGGGTACTTT TGATCAAGTA TGTATGCGGT TGTTGAGATA
      1207        1217        1227        1237        1247        1257
ATTCTTGGGC TCTATTGGAA TACCATGAGC CTGCATGTGT TGCTGGACGT ATTGACATGT
      1267        1277        1287        1297        1307        1317
TTGAAAAATT CTATTCTTTG CACTGTAGTC CACCTAAGCC ACCGACTAGG ACCACTTCAC
      1322
TTAAG
```

Fig. 6

Figure 7

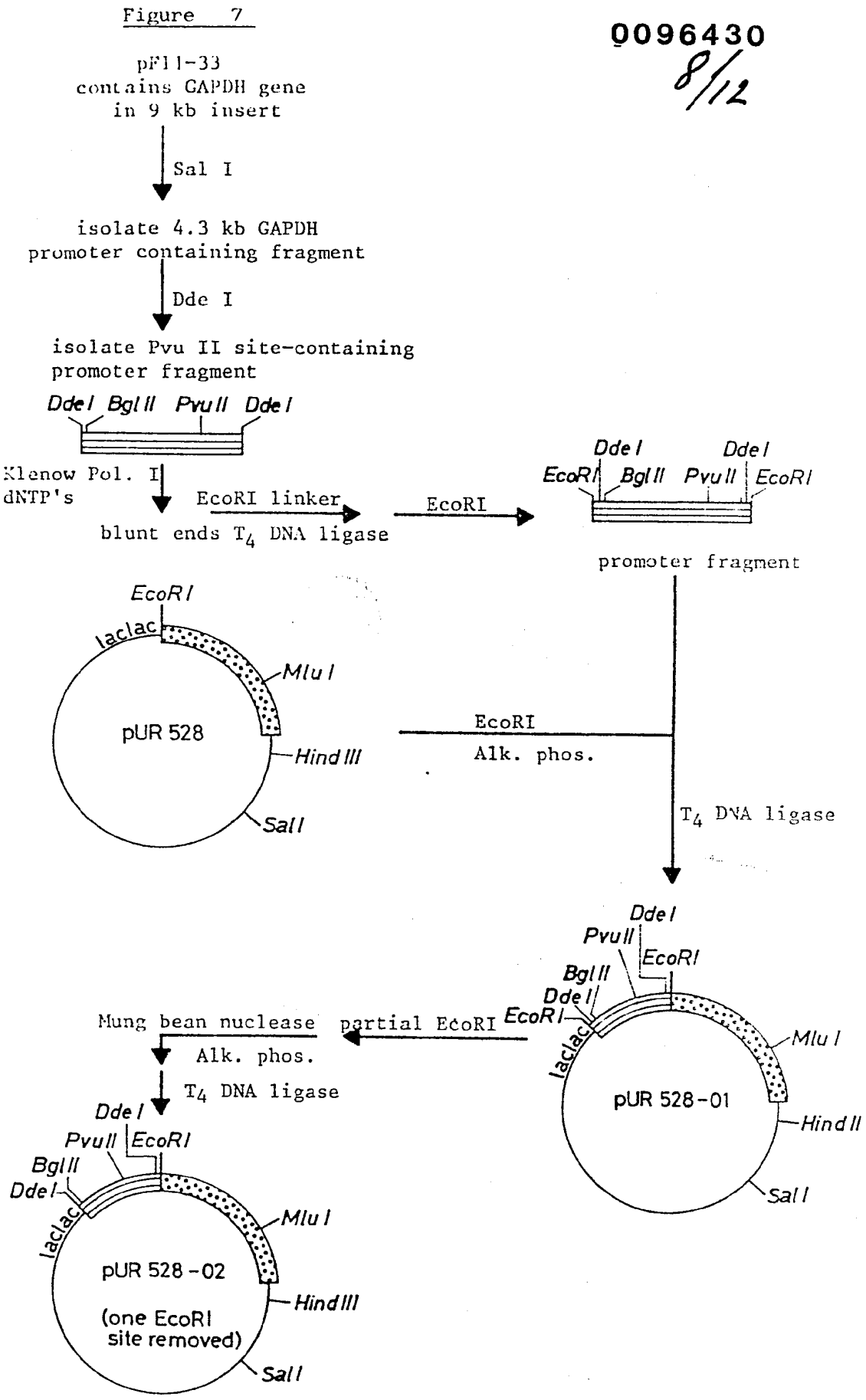

pF11-33
contains GAPDH gene
in 9 kb insert
Sal I
isolate 4.3 kb GAPDH
promoter containing fragment
Dde I
isolate Pvu II site-containing
promoter fragment
DdeI BglII PvuII DdeI
Klenow Pol. I
dNTP's
EcoRI linker
blunt ends T4 DNA ligase
EcoRI
DdeI DdeI
EcoRI BglII PvuII EcoRI
promoter fragment
EcoRI
laclac
MluI
pUR 528
HindIII
SalI
EcoRI
Alk. phos.
T4 DNA ligase
DdeI
PvuII
EcoRI
BglII
DdeI
EcoRI
laclac
MluI
pUR 528-01
HindII
SalI
Mung bean nuclease
Alk. phos.
T4 DNA ligase
partial EcoRI
DdeI
PvuII
EcoRI
BglII
DdeI
laclac
MluI
pUR 528-02
(one EcoRI
site removed)
HindIII
SalI

Figure 8

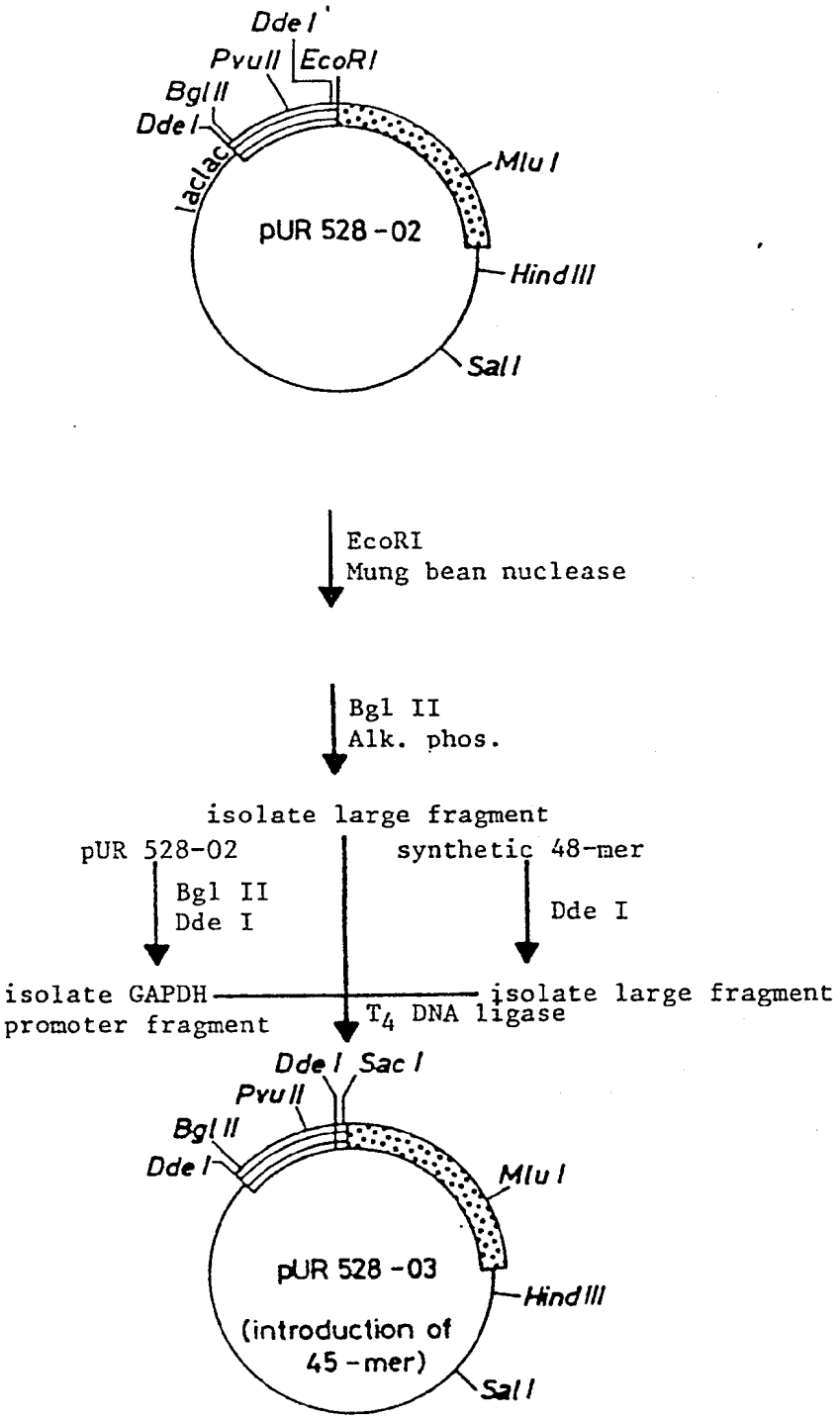

Dde I
PvuII
EcoRI
BglII
Dde I
laclac
MluI
pUR 528-02
HindIII
SalI
EcoRI
Mung bean nuclease
Bgl II
Alk. phos.
isolate large fragment
pUR 528-02
synthetic 48-mer
Bgl II
Dde I
Dde I
isolate GAPDH promoter fragment
T4 DNA ligase
isolate large fragment
Dde I
Sac I
PvuII
BglII
Dde I
MluI
pUR 528-03
(introduction of 45-mer)
HindIII
SalI

Figure 9

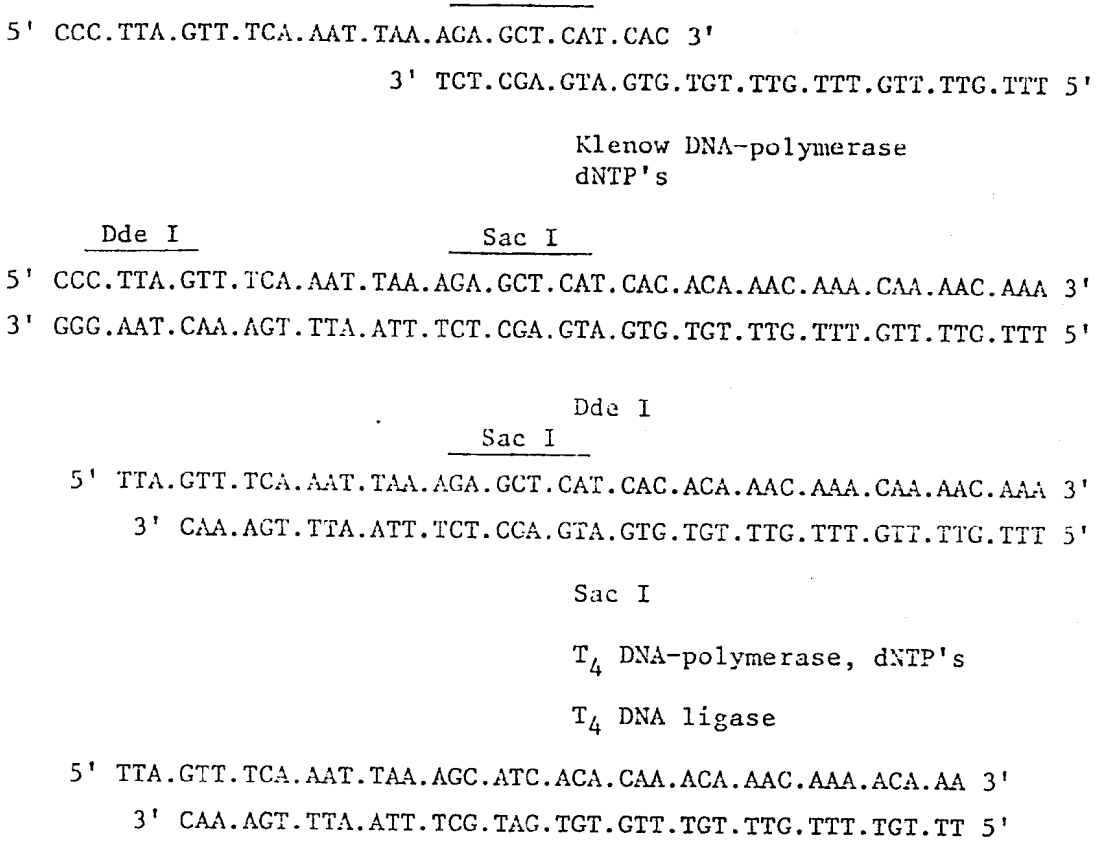
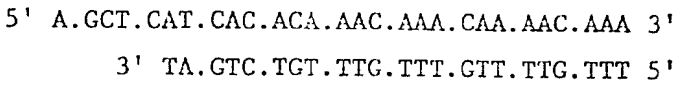
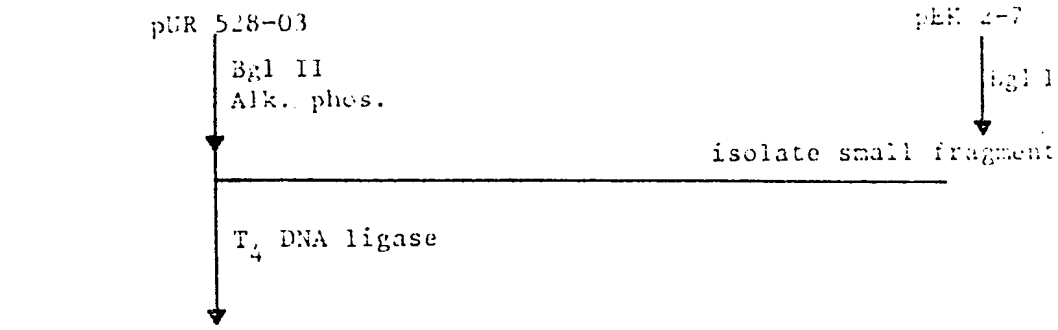


Fig. 7b




pUR 528-03
Bgl II
Alk. phos.
pEK 2-7
Bgl I
isolate small fragment
T4 DNA ligase

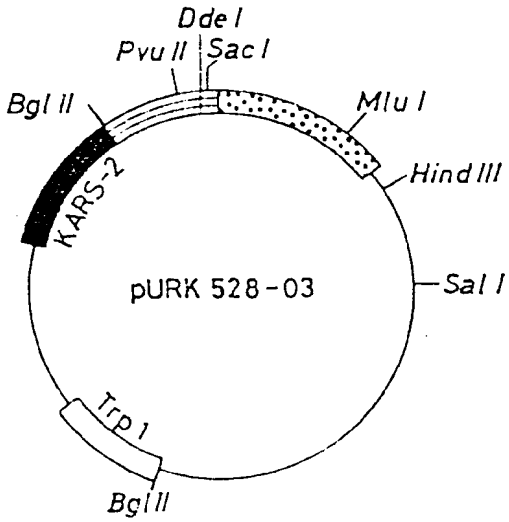

Dde I
Pvu II
Sac I
Bgl II
Mlu I
Hind III
KARS-2
pURK 528-03
Sal I
Trp 1
Bgl II

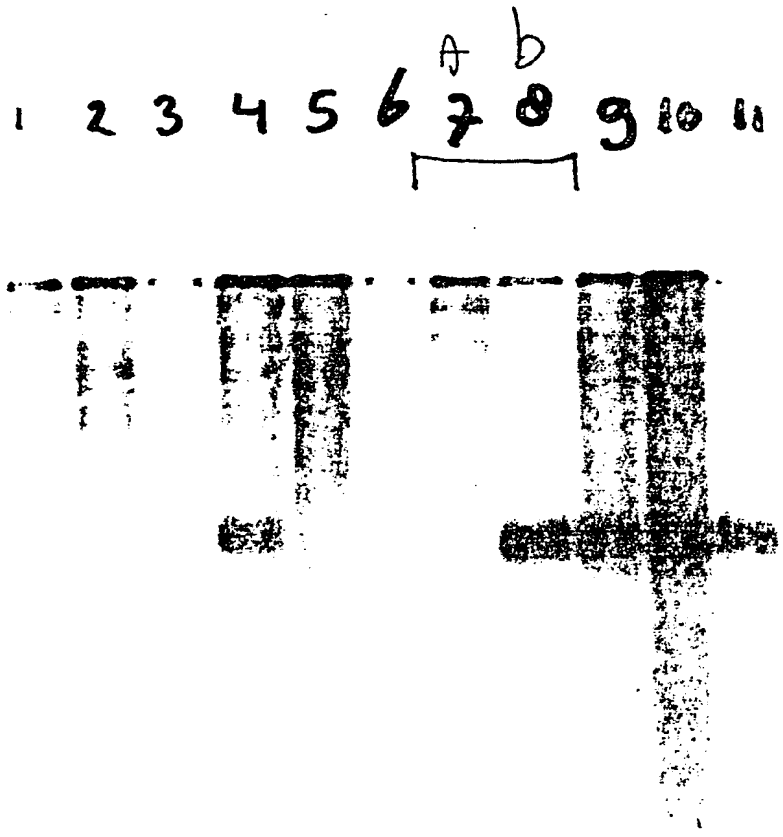
Figure 11

Analysis of ^{35}S-labeled proteins from K. lactis SD11 cells transformed with pURK 528-03.
K. lactis SD11 cells were grown in the presence of $^{35}SO_4^=$. The labeled cells were converted to protoplasts and the proteins were immunoprecipitated and analyzed on PAA-gels as described by L. Edens et al., Gene 18 (1982),1.

Lane 5:
Immunoprecipitated ^{35}-S-labeled proteins from K. lactis SD11 cells transformed with plasmid pEK2-7.

Lane 7:
Radioactively labeled marker proteins (Amersham)

Lane 8:
Immunoprecipitated ^{35}-S-labeled proteins from K. lactis SD11 cells transformed with plasmid pURK528-03.



Gist-Brocades nv patents and trademarks

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Trademarks
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Documentation
Drs. A.H.J.M. Sikken

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EUR-2362 Hu/io	015-79 2712	September 19, 1983.

Dear Sirs,

Re: European Patent Application No. 83200714.0.

We herewith inform you, in conformity with Rule 28(1) and (2) EPC, that on May 19, 1983, a culture of a microorganism has been deposited for the purpose of the invention described in the above-identified patent application with the Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands, under No. CBS 8092.

The deposited microorganism is a culture of the strain Kluyveromyces lactis SD 11 containing plasmid pUkK 528-02. The construction of said plasmid was identical to the construction of the plasmid pURK 528-03, with the exception that the plasmid in which the insertion took place was plasmid pUR 528-02 instead of pUR 528-03 (cf. page 35 and Figures 7 and 10 of the specification).

We take this opportunity to request that the following corrections in the specification may be made (Rule 88 EPC):

~~page 6, lines 37-38 should read: "For example, it is known from S. Das and G.P. Hollenberg, Current Genetics 6 (1982) 123-128, that plasmid PMP81 can be"~~

page 9, line 31: "OP_{610}nm" should read "OD_{610}nm"

page 9, line 33: "preheated" should read "pretreated"

~~page 10, line 11: "G12" should read "G21"~~

page 11, line 14: "dueg" should read "due"

2/...

Wateringseweg 1
P.O. Box 1
2600 MA Delft Holland

~~page 12, line 3: after "plasmid pL4" add "(cf. Example 3)"~~

page 12, line 12: "plamids" should read "plasmids"

~~page 12, line 22: "Kluyveromyces lactis lac4 should read "Kluyveromyces lactis trp1 lac4"~~

page 13, line 31: "consisting of" should read "comprising"

~~page 22, lines 6, 11 and 19: "pGB 181" should read "pGL2"~~

page 22, line 8: ~~"plasmid pACYC 184" should read "plasmid pACYC 177, Chang et al., J. Bacteriol. 134 (1978), 1141-1156,"~~

~~page 22, line 11: "G12" should read "G21"~~.

Yours faithfully,
Gist-Brocades N.V.

Dr. A.V. Huygens.

The request for correction is allowed under R. 88 EPC / with the exception of the deleted points /.
THE HAGUE, 18. 10. 83
RECEIVING SECTION
P. J. MASSAAR

European Patent Office

EUROPEAN SEARCH REPORT

0096430
Application number

EP 83 20 0714

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
X	PATENTS ABSTRACTS OF JAPAN, vol. 5, no. 98(C-60)(770), 25th June 1981, & JP - A - 56 39099 (MITSUBISHI KASEI KOGYO K.K.) 14-04-1981 * Whole abstract *	9,32	C 12 N 15/00 C 12 N 1/16 C 12 N 9/60 C 12 N 9/38 // C 12 R 1/645
A	PATENTS ABSTRACTS OF JAPAN	1	
X	EP-A-0 048 081 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) * Whole document *	9,32	
X	GENE, vol. 15, 1981, pages 157-166, Elsevier/North-Holland Biomedical Press CHU-LAI HSIAO et al.: "Characterization of a yeast replication origin (ars2) and construction of stable minichromosomes containing cloned yeast centromere DNA (CEN3)" * Whole document *	9,32	TECHNICAL FIELDS SEARCHED (Int. Cl. ³) C 12 N C 12 P C 12 R
X	EP-A-0 045 573 (THE BOARD OF TRUSTEES OF LELAND STANFORD JUNIOR UNIVERSITY) * Whole document *	9	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21-07-1983	DESCAMPS J.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503 03.82

European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 83 20 0714

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
X	NATURE, vol. 293, 29th October 1981, pages 717-722, Macmillan Journals Ltd. R.A. HITZEMAN et al.: "Expression of a human gene for interferon in yeast" * Whole document *	9	
D,X	GENE, vol. 10, 1980, pages 347-356, Elsevier/North-Holland . Biomedical Press R.C. DICKSON: "Expression of a foreign eukaryotic gene in Saccharomyces cerevisiae: beta-galactosidase from Kluyveromyces lactis" * Whole document *	11	
P,X	GB-A-2 094 341 (MITSUBISHI CHEMICAL INDUSTRIES LTD.) * Page 3, lines 31-37 *	9,32	TECHNICAL FIELDS SEARCHED (Int. Cl. ³)
P,A		1	
P,X	EP-A-0 057 350 (COLLABORATIVE RESEARCH INC.) * Page 42, line 29 - page 51, end; claims 38-40 *	9	
P,X	EP-A-0 073 635 (A.J. KINGSMAN et al.) * Whole document *	9,32	

-/-

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21-07-1983	DESCAMPS J.A.

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O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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European Patent Office

0096430
Application number
EP 83 20 0714

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
P,X	EP-A-0 060 057 (BOARD OF REGENTS OF UNIVERSITY OF WASHINGTON) * Whole document *	9	
A	JOURNAL OF BACTERIOLOGY, vol. 147, no. 1, July 1981, pages 155-160, N. GUNGE et al.: "Intergeneric transfer of deoxyribonucleic acid killer plasmids, pGKI1 and pGKI2, from Kluyveromyces lactis into Saccharomyces cerevisiae by cell fusion" * Whole document *		
			TECHNICAL FIELDS SEARCHED (Int. Cl. ³)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21-07-1983	DESCAMPS J.A.

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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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